UNIVERSIDAD AUTÓNOMA DE MADRID

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“PAPEL DE Cot/tpl-2 EN INMUNIDAD INNATA Y MIGRACIÓN CELULAR”

CRISTINA RODRÍGUEZ RIVERO

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"PAPEL DE Cot/tpl-2 EN INMUNIDAD INNATA Y MIGRACIÓN CELULAR"

Memoria de Tesis Doctoral presentada para optar al grado de Doctor por la Licenciada en Ciencias Biológicas

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CERTIFICA:

Que Cristina Rodríguez Rivero, Licenciada en Ciencias Biológicas, ha realizado bajo mi dirección la Tesis Doctoral titulada "Papel de Cot/tpl-2 en inmunidad innata y migración celular".

Considero que tanto el trabajo experimental realizado como las conclusiones del mismo reúnen las condiciones exigidas para optar al grado de Doctor.

Y para que conste y a efectos oportunos, firmo el presente certificado en Madrid a 18 de Noviembre de 2006

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La memoria es el bosque donde los recuerdos se pierden.
La imaginación es la memoria de recuerdos olvidados

Gonzalo Suárez.

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AGRADECIMIENTOS

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ÍNDICE

RESUMEN/SUMMARY

La MAP quinasa quinasa quinasa (MKKK) Cot/tpl-2 tiene relevancia en fenómenos de inmunidad innata. Nosotros hemos demostrado que Cot/tpl-2 es la única MKKK que media la activación de la ruta de ERK1/ERK2 en respuesta a LPS e IL-1. La activación de Cot/tpl-2 por estos estímulos requiere su disociación del complejo inactivo p105 NF-κB/Cot y permite que Cot/tpl-2 sea degradada por el proteasoma. El mecanismo de disociación de Cot/tpl-2 es dependiente de la proteína TRAF 6. Además, la activación de Cot/tpl-2 requiere una actividad tirosina quinasa de la familia de Src. Cot/tpl-2 fue identificada como un oncogen en una forma modificada/truncada, revelando el potencial oncogénico de la proteína. En este trabajo también hemos demostrado que, fisiológicamente, Cot/tpl-2 también juega un papel en migración celular regulando varios de los pasos implicados en este proceso. La expresión de la forma truncada/oncogénica de Cot/tpl-2 induce un incremento de la migración e invasión celular y una disminución de la adhesión celular mientras que el bloqueo de la actividad endógena de Cot/tpl-2 produce una reducción del 70-80% en la migración celular. Establecemos también que Cot/tpl-2 regula la migración gracias a su capacidad para modular la actividad de Rho y la expresión de COX-2. Todos estos datos sirven para esclarecer el papel de Cot/tpl-2, en los fenómenos de invasión y migración celular, apoyando la idea de que la invasión y migración celular comparten bases moleculares comunes.

The MAP kinase kinase kinase Cot/tpl-2 has been related to immune processes. Here we show that Cot/tpl-2 is the sole MKKK responsible for ERK1/ERK2 activation in response to IL-1 and LPS. LPS or IL-1-mediated-Cot/tpl-2 activation requires its dissociation from the inactive p105 NFκB-Cot/tpl-2 complex which in resting cells protects Cot/tpl-2 from degradation. The Cot/tpl-2 dissociating mechanism is dependent on the activity of the transducer protein TRAF6. Besides a tyrosine kinase activity from the Src family is required for Cot/tpl-2 activation. Cot/tpl-2 was identified as an oncogene in a truncated/modified form, revealing the oncogenic potential of the protein. Here, we demonstrate that Cot/tpl-2 regulates cell invasion by modulating different steps involved in the process. Cells overexpressing Cot/tpl-2 truncated exhibit a statistically significant 1.5 fold enhanced invasion, a 2-4-fold decrease in the adhesion and a 1.8-fold increase in the migration of the cells respect to control cells. On the other hand, the blockage of endogenous Cot/tpl-2 reduces a 70-80% cell migration. Furthermore, we demonstrate that Cot/tpl-2 ability of regulating cell migration is due to its capacity to regulate COX-2 expression and Rho activity. Inflammatory and invasiveness processes share some common molecular basis and here we show that Cot/tpl-2 activity is also involved in cell invasion.

ABREVIATURAS

ABIN-2: Inhibidor A-20 de unión a NF-κB

ADN: Ácido desoxi-ribonucleico

ADN-CpG: ADN rico en citidina e guanidina

AKT/PKB: Proteína quinasa B

AP-1: Proteína activadora 1

ARN: Ácido ribonucléico

ARNm: ARN mensajero

ARNsi: ARN silenciador

ARP 2/3: Proteínas relacionadas con la actina

COX-2: Ciclooxigenasa-2

CREB: Proteína de unión al elemento de respuesta de cAMP

DD: Dominio de muerte

ECM: Matriz extracelular

ERK: Quinasa regulada por señales extracelulares

E2F: Factor de transcripción 2F

FAK: Quinasa de adhesiones focales

FBS: Suero fetal bovino

GAP: Proteína activadora de la GTPasa

GDI: Inhibidor del intercambio de nucleótido guanina

GPI: glicofosfoinositósidos

GEFs: Factores intercambiadores de nucleótidos de guanidina

Gö 6850: Bisindolilmaleimida I

Herb A: Herbimicina A

HTLV-1: Virus de la leucemia tipo 1

IκB: Inhibidor del factor nuclear kappa B

IKK: Quinasa del complejo IκB

IL: Interleuquina

IL-1RAcP: Proteína accesoria del receptor de IL-1

IRAK: Quinasa asociada al receptor de IL-1

JNK: Quinasas N-terminales de c-Jun

LBP: Proteína de unión al LPS

LPS: Lipopolisacárido

MAPK: Proteína quinasa activada por mitógeno

MIP1: Proteína inductora de la activación de macrófagos

MKK1: MAP quinasa quinasa 1

MKKK: MAP quinasa quinasa quinasa

MLC: Cadena ligera de la miosina

MLCK: Quinasa de la cadena ligera de la miosina

MMP: Metaloproteasa

MMTV: Virus de tumores de mama en ratones

MyD88: Proteína adaptadora de la respuesta de diferenciación primaria Mieloide

NEMO/IKKγ: Modulador esencial de NF-κB

NFAT: Factor nuclear de activación de células T

NF-κB: Factor nuclear kappa B

NIK: Quinasa inductora de NF-κB

PAI-1: Inhibidor del activador del plasminógeno 1

PAMPs: Patrones moleculares asociados a patógenos

15d-PGJ$_2$: 15-deoxi-Δ12,14-prostaglandina J_2

PGE$_2$: Prostaglandina E_2

PI3K: Fosfatidil-inositol-3-quinasa

PKC: Proteína quinasa C

PMA: Forbol 12-miristato 13-acetato

PPAR-γ : Receptor -γ activado por factores proliferadores peroxisomales

PP1: Proteína fosfatasa 1

PP2A: Proteína fosfatasa 2A

pSR: pSuperRetro

ROCK: Rho quinasa

Src: Proteína quinasa del Sarcoma de Rous

TAB: Proteína de unión a TAK1

TAK1: Quinasa activada por los receptores Toll

TH: T colaboradores (linfocitos)

Thr: Treonina

TIMP: Inhibidores tisulares de las metaloproteasas

TIR: Dominio del receptor Toll/interleuquina-1

TIRAP/MAL: Proteína asociada al dominio TIR

TLR: Receptor tipo Toll

TNF-α: Factor de necrosis tumoral α

TOLLIP: Proteína de interacción con Toll

TRAF-6: Factor 6 asociado al receptor de TNF

Tyr: Tirosina

WASP: Proteína del síndrome de Wiskott-Aldrich

WT: Salvaje

INTRODUCCIÓN

1. Inmunidad innata y adquirida.

Los organismos superiores están constantemente expuestos a posibles infecciones por parte de los microorganismos presentes en su entorno. Para hacer frente a estas infecciones el sistema inmune de los vertebrados ha desarrollado diversas estrategias. Por un lado, los organismos presentan una respuesta rápida que constituye una primera línea de defensa ante los posibles patógenos (respuesta innata). Existe además una respuesta más tardía (que implica la participación de células B y T) caracterizada por la producción, mediante reordenaciones génicas, de anticuerpos y receptores de membrana (por parte de las células B y T respectivamente) capaces de reconocer de forma específica epítopos pertenecientes a distintos agentes patogénicos (respuesta adquirida) [Consultar para revisión (Fearon 1996)]. Aunque históricamente siempre se ha concedido una mayor importancia al estudio de la inmunidad adquirida por ser más específica, descubrimientos recientes han permitido demostrar que la respuesta innata posee cierta especificidad. Esta especificidad, que permite al sistema inmune distinguir entre epítopos del propio organismo y los correspondientes a organismos patógenos externos, es esencial para la posterior activación de la respuesta adquirida (Medzhitov 2001).

2. Receptores en respuesta inmune innata. Toll like receptor (TLR) y receptor de IL-1.

2.1. Receptores Tipo Toll.

La capacidad del sistema inmune para discriminar entre los antígenos propios y los pertenecientes a patógenos exógenos está determinada por una familia de receptores evolutivamente conservados denominados receptores tipo Toll (Toll-like receptors, TLRs). Estos receptores, activados en respuesta a distintos componentes de los microorganismos, tienen un papel esencial en la respuesta primaria a organismos patógenos e inducción de la respuesta adquirida (Barton 2002 a; Pasare 2004; Barton 2002 b; Masuda 2002).

Los TLR fueron descubiertos como proteínas homólogas al receptor Toll de *Drosophila melanogaster* (Rock FL 1998). La proteína Toll, que juega un papel esencial en el establecimien-

to de la polaridad dorsoventral durante la embriogénesis (Hashimoto 1988), está relacionada con la respuesta inmune innata del insecto ante infecciones fúngicas (Lemaitre B 1996). En mamíferos, se han identificado al menos 11 miembros de la familia de TLR aunque sólo del TLR1 al TLR10 se han identificado en humanos. Estos receptores son capaces de reconocer una gran variedad de patrones moleculares asociados a patógenos (PAMPs) (Consultar Tabla 1).

Los TLRs son glicoproteínas integrales de membrana que presentan homología de secuencia, en su región citoplasmática, con la familia génica del receptor de IL-1 (Heguy 1992) aunque difieren en su región extracelular. Los TLRs presentan, en su región extracelular, motivos ricos en Leucina (LRR) en lugar de los dominios tipo inmunoglobulina del receptor de IL-1. La región citoplasmática de 200 aminoácidos, presente tanto en TLR como en el receptor de IL-1, conocida como dominio TIR (dominio Toll/IL-1R) presenta 3 regiones de secuencia altamente conservada (20-30%) y homólogas para los distintos TLRs. Esta región, es de gran importancia para la transducción de la señal (O'Neill 2000). Así pues, una mutación en la región TIR del gen para TLR 4 induce una respuesta deficiente al lipopolisacárido (LPS), componente mayoritario de la pared de las bacterias Gram negativas (Poltorak 1998).

TLR	***Ligando***	***Origen del Ligando***	***Moléculas asociadas***
TLR1	Triacil péptidos	Bacterias, Micobacteria	TLR 2
TLR2	Lipoproteínas y lipopéptidos Peptidoglicano, MALP-2	Varios patógenos Bacterias Gram-positivas	TLR 1 y 6
TLR3	DNAs de cadena doble, poli IC	Virus	
TLR4	Lipopolisacárido (LPS), Taxol	Bacterias Gram-negativas Plantas	CD14, MD-2, LBP
TLR5	Flagelina	Bacterias	
TLR6	Diacil lipopéptidos y Zimosan Acido lipoteicoico	Micoplasma y Hongos Bacterias Gram-positivas	TLR2
TLR7	DNA de cadena simple	Virus	
TLR8	DNA de cadena simple	Virus	
TLR9	DNA con secuencias CpG	Bacterias y Virus	
TLR10	Desconocido		
TLR11	Desconocido		

Tabla 1. Receptores tipo Toll y ligandos que los activan.

En líneas generales, el mecanismo de transducción de señales de los TLRs es compartido por el receptor de IL-1 e implica la activación de la ruta de señalización intracelular de NF-κB (Liu 2002) y de las cascadas de las distintas MAPKs (Cario 2000).

La activación de la ruta de NF-κB requiere la participación de distintos componentes considerados esenciales para la transducción de la señal, entre los que se encuentran: la proteína adaptadora de la respuesta de diferenciación primaria mieloide (MyD88) (Muzio, Ni et al. 1997; Burns, Martinon et al. 1998; Medzhitov, Preston-Hurlburt et al. 1998; Kawai, Adachi et al. 1999), el adaptador TOLLIP (Toll- interacting protein) (Burns, Clatworthy et al. 2000), la quinasa asociada al receptor de IL-1 (IRAK) (Suzuki N 2002) y el adaptador TRAF6 (Gohda 2004).

La deleción dirigida de los genes de TRAF6 y MyD88 han permitido establecer su papel dentro de los mecanismos de señalización de las rutas del receptor de IL-1 (IL-1R) y TLRs (Burns, Martinon et al. 1998; Kawai, Adachi et al. 1999; Lomaga 1999). MyD88 posee dos dominios de interacción proteína-proteína, un dominio de muerte (death domain, DD) en su extremo N-terminal y una región TIR en su extremo carboxilo. Mientras que la región TIR del extremo carboxilo terminal permite a MyD88 interaccionar con el receptor TLR o IL-1R, su dominio de muerte interacciona con la quinasa IRAK reclutándola al complejo (Muzio, Ni et al. 1997; Wesche 1997; Medzhitov, Preston-Hurlburt et al. 1998). La proteína TOLLIP, es también capaz de interaccionar con el receptor y reclutar a IRAK. Una vez reclutada IRAK al complejo, se autofosforila y asocia con TRAF6 (Cao 1996), este adaptador induce a su vez la activación de la quinasa TAK1.

La quinasa TAK1, que se encuentra asociada a moléculas adaptadoras como TAB 1, 2 y 3 (Ge 2002; Shim 2005; Takaesu 2003), es capaz de inducir la activación de distintas rutas de MAPK. TAK1 además, regula la actividad quinasa de las unidades catalíticas del complejo IKK lo que permite la activación de NF-κB (Wang 2001)(Figura 1).

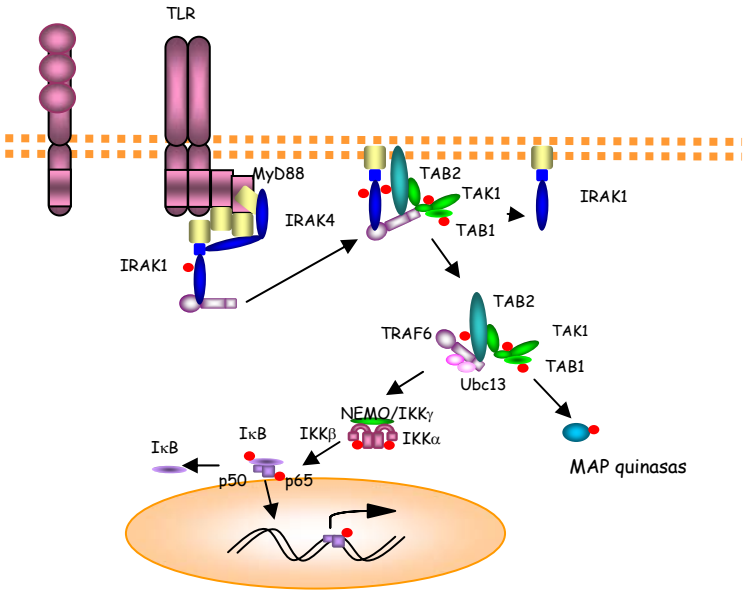

Figura 1. Activación de los TLRs/IL-1R.

En células no estimuladas, el factor de transcripción NF-κB se encuentra acomplejado con un inhibidor formado por dos subunidades (α y β) denominado I*k*B que impide su translocación al núcleo. La degradación previa por medio del proteasoma de este inhibidor específico es esencial para la completa activación de NF-κB (Chen 2005). Para que se produzca la degradación de las proteínas que conforman el inhibidor I*k*B ambas subunidades deben ser fosforiladas en dos residuos de serina específicos. Esta fosforilación es mediada por la actividad quinasa del complejo IKK.

El complejo IKK está formado por 2 subunidades catalíticas (IKKα e IKKβ), que presentan cierto solapamiento en alguna de sus funciones, y una subunidad reguladora denominada IKKγ o NEMO (Modulador esencial de NF-κB). Las subunidades catalíticas de está quinasa se consideran esenciales en la activación de NF-κB, además NEMO tiene un papel de relevancia en esta ruta de señalización intracelular y es requerida para la correcta estimulación de NF-κB en respuesta a LPS e IL-1 (Yamaoka 1998; May 2000; Rudolph, Yeh et al. 2000).

Así pues, el mecanismo de activación de NF-κB y las distintas MAP quinasas implica el reclutamiento/activación de estos componentes de modo secuencial siguiendo el esquema MyD88/IRAK/TRAF6/TAK1, si bien es cierto que algunos TLR utilizan mecanismos alternativos

con proteínas accesorias específicas. Así estudios realizados en el ratón deficiente para el gen MyD88 permitieron demostrar que, en el caso de los TLRs 4 y 3, existen dos mecanismos de transducción de señales para la activación de NF-κB y las MAP quinasas, uno de ellos es dependiente de MyD88 y otro independiente. En éste la proteína TIRAP (también denominada Mal) es la encargada de activar la ruta de señalización intracelular (Golenbock 2001; Horng 2001; Fitzgerald 2001; Yamamoto, Sato et al. 2002).

2.2. Receptor de IL-1.

La familia de los receptores de IL-1 engloba una serie de proteínas transmembrana y proteínas solubles caracterizadas por poseer dominios extracelulares tipo Inmunoglobulina (Ig). Estos receptores suelen formar un complejo con una proteína accesoria o co-receptor que carece de sitio de unión a ligando. Para el receptor de IL-1, esta molécula se denominada proteína accesoria del receptor de IL-1 o IL-1RAcP (Greenfeder 1995).

El receptor de IL-18 (denominado anteriormente IL-1 Rpr) también posee una molécula co-receptora específica, IL18Rh, que resultó ser un homólogo estructural y funcional de IL-1RAcP (Parnet 1996).

Además existen una serie de receptores huérfanos que presentan características similares a los receptores de IL-1 y IL-18 y que se engloban dentro de esta familia. Todos estos receptores se caracterizan, además de por la presencia de los dominios extracelulares tipo Ig, por la existencia de una región TIR en su dominio citoplasmático que induce la activación de las rutas de NF-κB y las MAPKs (mediante un mecanismo que comparten con los TLR).

La unión de receptores de interleuquinas a sus ligandos específicos en la superficie de las células T y B, fibroblastos o células epiteliales, es requerida para la inducción de la respuesta inmune adquirida. Así, IL-1 es requerida para la activación de la respuesta TH1 (en la que actúa de forma sinérgica con la IL-2) (Shibuya K 1998; Quill 1989) y juega un papel esencial en el desarrollo de los linfocitos TH2 (Manetti 1994), demostrándose que las citoquinas IL-1 y IL-18 (Dinarello 1996; Dinarello 1999) no son exclusivamente simples mediadores de la respuesta innata.

3. MAP quinasas, quinasas, quinasas, en respuesta inmune innata: El papel de Cot/tpl-2.

La activación de las distintas MAPKs constituye un paso clave para la transmisión de la señal generada por la activación de múltiples receptores.

Las distintas MAP quinasas pueden clasificarse en: ruta de ERK1/ERK2, ruta de JNK2/JNK3, ruta de las p38 MAP quinasas (α, β, γ y δ) y ruta de ERK5 y presentan un mecanismo de activación altamente conservado desde levaduras a mamíferos.

Estas quinasas son el último eslabón de módulos de tres componentes con actividad quinasa donde el primer componente es una MAP quinasa quinasa quinasa (MKKKs) (Figura 2). Las MKKKs, son activadas por medio de otras quinasas de serina/treonina, tirosina quinasas, o por interacción con proteínas de unión a GTP (como los miembros de la familia de Ras o Rho). Tras su activación, las MKKKs son las encargadas de fosforilar (en residuos de serina y/o treonina) y activar al siguiente componente del modulo, las MKKs. Cada MKKK es capaz de unirse y activar distintas MKKs mientras que una misma MKK puede ser activada por quinasas distintas lo que implica que las MKKs son puntos de convergencia de las distintas señales celulares como demuestra el hecho de que, en mamíferos, el número de MKKKs doble al número de MKKs (14 MKKKs distintas por sólo 7 MKKs).

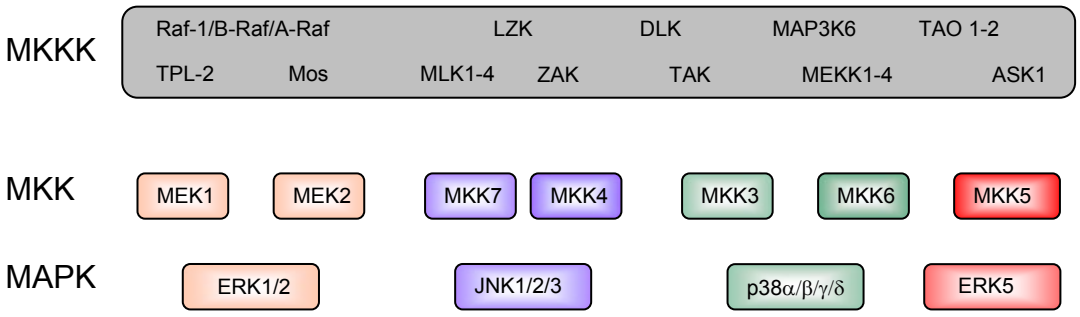

Figura 2. Módulos de MAP quinasas.

Las MKKs son quinasas duales que reconocen y fosforilan los motivos Thr-X-Tyr (treonina-aminoácido-tirosina) del lazo de activación de las MAP quinasas mediante una asociación altamente específica (Ahn 1991). Una vez activadas, las MAP quinasas son capaces de fosforilar a sus substratos específicos entre los que encontramos diversos factores de transcripción, otras quinasas, fosfolipasas, proteínas implicadas en el metabolismo celular y proteínas asociadas al citoesqueleto.

En el sistema inmune existen numerosas MKKKs implicadas en la activación de las rutas de las MAPKs en respuesta a diversos estímulos entre las que se encuentra Cot/tpl-2.

3.1. Cot/tpl-2. El estudio del ratón knock-out para el gen de tpl-2, el homólogo murino de Cot, permitió establecer que, en macrófagos, Cot/tpl-2 es responsable de la activación de la ruta de ERK1/ERK2 en respuesta a LPS, mientras que las rutas de las MAP quinasas JNK y p38γ no se ven afectadas por la ausencia de Cot/tpl-2 (Dumitru, Ceci et al. 2000). La producción de TNF-α, en macrófagos, está mediada por la vía Cot/tpl-2→ERK1/ERK2. Así, el ratón knock-out es resistente a la patología inducida por LPS/D-Galactosamina debida a la baja producción de TNF-α por los macrófagos. Estos datos relacionan a Cot/tpl-2 con la inmunidad innata (Dumitru, Ceci et al. 2000).

Está establecido que en células no estimuladas, Cot/tpl-2 forma un complejo multiproteíco estable e inactivo con la subunidad p105 de NF-κB entre otras proteínas (Belich, Salmeron et al. 1999; Beinke, Deka et al. 2003). De hecho, recientemente se ha descrito que la proteína ABIN-2 es también esencial para mantener la estabilidad del complejo multiproteíco y la activación de ERK1/ERK2 en respuesta a LPS (Lang 2004; Papoutsopoulou, Symons et al. 2006).

La estimulación de la célula induce la activación del complejo IKK y promueve, por medio de la subunidad IKKβ, la fosforilación de p105 NF-κB que es rápidamente fragmentado por el proteasoma dando lugar al péptido p50 NF-κB (una de las subunidades parte del factor de transcripción NF-κB). Como consecuencia de esta degradación, Cot/tpl-2 es liberado del complejo lo que le permite transducir la señal activadora (Waterfield, Zhang et al. 2003; Beinke, Robinson et al. 2004; Waterfield, Jin et al. 2004). Esta liberación del complejo permite que Cot/tpl-2 sea posteriormente, y una vez activada, degradada por el proteasoma (Figura 3).

El gen humano de Cot fue identificado a partir de una forma modificada en su extremo 3´ que originaba la expresión de una proteína modificada/truncada. En esta proteína, los últimos 69 aminoácidos, correspondientes al extremo carboxilo terminal, se encuentran sustituidos por 18 aminoácidos sin ninguna homología de secuencia (Miyoshi, Higashi et al. 1991).

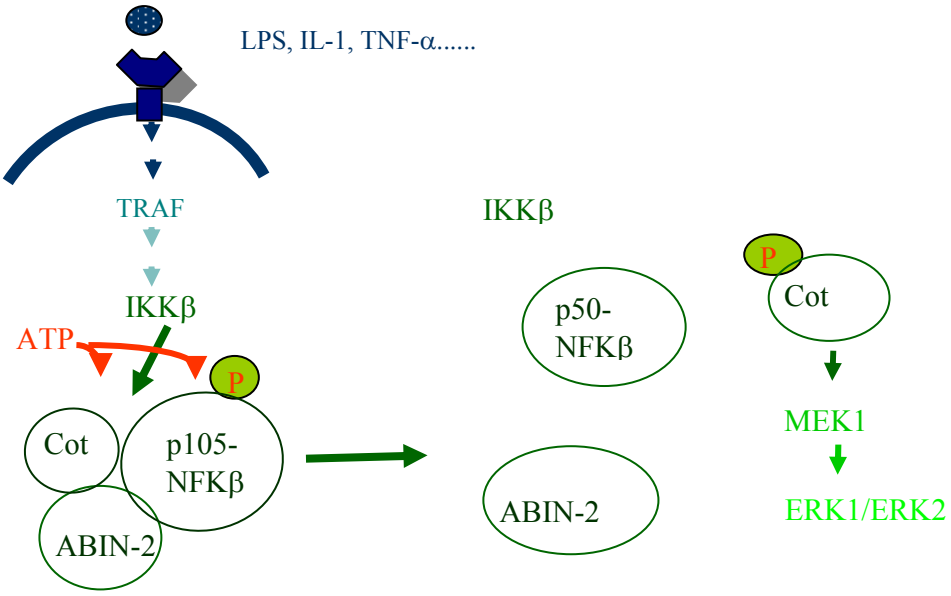

Figura 3. Activación de Cot.

Al igual que Cot su homólogo murino, el gen tpl-2, fue identificado en una versión modificada/truncada (Ceci, Patriotis et al. 1997). Estas modificaciones desenmascaran la capacidad transformante de esta MKKK.

Por otra parte, se ha demostrado que una sobreexpresión de la forma proto-oncogénica de Cot/tpl-2 es también capaz de conferir un fenotipo transformante a líneas celulares establecidas (Chan, Chedid et al. 1993; Chiariello, Marinissen et al. 2000; Gándara 2003) .

Además se han descrito, en la forma salvaje de Cot/tpl-2, dos sitios alternativos de la iniciación de la traducción denominados M(1) y M(29) (Aoki, Hamada et al. 1993). La forma M(29) de Cot/tpl-2 salvaje, no contiene los primeros 29 aminoácidos presentes en la forma proteica a la que da lugar M(1) y presenta una vida media 2 o 3 veces inferior a la forma M(1) (Aoki, Hamada et al. 1993).

El dominio carboxilo terminal de Cot/tpl-2 salvaje contiene una secuencia de aminoácidos denominada degrón, (EMLKRQRSLYIDLGALAGYFNL) que permite que la proteína sea reconocida por el proteasoma para su posterior degradación. Este degrón, cuando se fusiona a otras proteínas, confiere inestabilidad y hace que la vida media de la proteína salvaje de Cot/tpl-2 sea unas 2,6 veces inferior a la de su forma truncada. Por otro lado, la deleción de 44 aminoácidos del extremo carboxilo terminal de la forma M1 de Cot/tpl-2 salvaje, produce también un incremento en la actividad específica de Cot/tpl-2 de 3,8 veces con respecto a su actividad basal (medida como activación de MKK1-ERK1/ERK2), lo que indica que el extremo carboxilo de Cot/tpl-2 actúa además como un dominio autoinhibitorio de la actividad específica en la forma salvaje de la proteína (Gándara 2003) (Figura 4).

De hecho, el extremo carboxilo terminal de tpl-2 es capaz de inhibir la actividad quinasa de la forma truncada de tpl-2 en ensayos *in vitro* (Ceci, Patriotis et al. 1997).

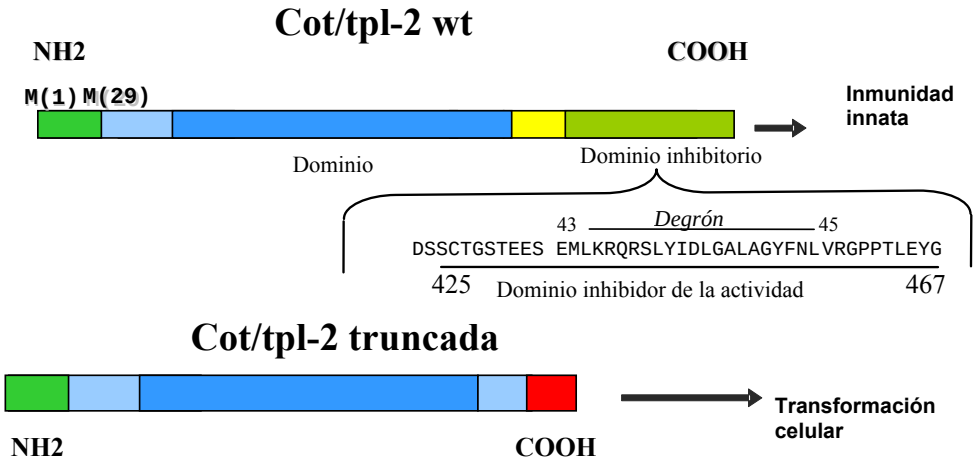

Figura 4. Proteína Cot/tpl-2.

Al sobreexpresar, tanto la forma salvaje de Cot/tpl-2 como su forma truncada, se produce una activación de distintas MAPKs como ERK1/ERK2, JNK (Salmeron, Ahmad et al. 1996), p38γ y ERK5 (Chiariello, Marinissen et al. 2000). Como consecuencia de la activación de las distintas MAPKs, se produce a su vez la activación de diversos factores transcripcionales como AP-1, NFAT y NF-κB (Ballester, Velasco et al. 1998; Chiariello, Marinissen et al. 2000; Kane, Mollenauer et al. 2002; Hammerman, Fox et al. 2004). De hecho, se ha propuesto que la capacidad de Cot/tpl-2 de producir transformación celular se debe a su capacidad para activar a AP-1 (Chiariello, Marinissen et al. 2000).

Tipo de mutación	Línea celular/tejido/alteración	Referencia
reorganización del gen de Cot/tpl-2 en 3´	Células de Hámster SHOK. Transfección in vitro de DNA humano.	*Miyoshi (1991)*
reorganización del gen de Cot/tpl-2 en 3´	Células T murinas. Inserción del virus de la leucemia de Molovni	*Patriotis (1993)*
reorganización del gen de tpl-2 en 3´	Inserción del virus de Tumor mamario de ratón.	*Erny (1996)*
reorganización del gen de Cot en 3´	Adenocarcinoma humano primario	*Clark (2004)*
Amplificación génica del locus de Cot/tpl-2	Cáncer de mama en humanos	*Sourvinos (1999)*
Modificaciones postraduccionales	Infección de células humanas por el virus de la leucemia tipo-I	*Babu (2006)*
Desconocida	leucemia granular linfocítica	*Christoforidou (2004)*
Desconocida	Células malignas humanas Hodgkin/Reed-Sternberg	*Eliopoulos (2002)*

Tabla 2. Alteraciones inducidas por Cot/tpl-2.

Se ha descrito una expresión anormal de Cot/tpl-2 en diversos tipos de células transformadas y dichas modificaciones en Cot/tpl-2, han sido propuestas como posibles responsables de la transformación celular (Tabla 2).

Por ejemplo, la ya citada reorganización del extremo 3´ de la región terminal del gen humano de Cot/tpl-2, induce transformación en células primarias murinas (Miyoshi, Higashi et al. 1991). La inserción del virus de la leucemia de Moloney en el gen tpl-2, que también produce una reorganización en la región terminal del extremo 3´ del gen tpl-2, juega un papel esencial en la progresión de linfomas de células T (Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993). De modo similar, la inserción del virus de tumores de mama en ratones (MMTV) en el último intrón del tpl-2, está asociada con la transformación celular en células de glándula mamaria (Erny, Peli et al. 1996) y, según datos más recientes, la modificación de la región 3´ también aparece en tumores de adenocarcinoma primarios humanos (Clark, Reynolds et al. 2004).

También se ha encontrado, en ciertos cánceres de mama humanos, una amplificación génica del locus de Cot/tpl-2 sin modificación alguna en su secuencia (Sourvinos, Tsatsanis et al. 1999). Además, se ha establecido una correlación entre los altos niveles de ARN mensajero (ARNm) de Cot y el desarrollo de la leucemia granular linfocítica (Christoforidou, Papadaki et al. 2004).

Más recientemente, se ha descrito que la infección de células T humanas por el virus de la leucemia tipo 1 (HTLV-1) induce la expresión inusualmente estable de la forma M(29) de Cot salvaje, probablemente debida a modificaciones postraduccionales en la proteína (Babu, Jin et al. 2006). Por otro lado, las células malignas humanas de Hodgkin/Reed-Sternberg también presentan unos niveles anormalmente altos de proteína de Cot/tpl-2, aunque se desconoce que tipo de modificación presenta el gen y/o la proteína (Eliopoulos, Davies et al. 2002).

Todos estos datos indican que la proteína Cot/tpl-2 es esencial en la activación de rutas de señalización en respuesta inmune y que una desregulación de su actividad endógena está implicada en procesos de transformación celular y formación de tumores.

4. Migración e invasión celular.

La invasión celular es un proceso fisiológico que permite a las células infiltrarse o penetrar a través de los vasos sanguíneos o barreras tisulares (como la matriz extracelular y el estroma intersticial). Es un proceso secuencial y complejo que engloba fenómenos como la modificación de la adhesión celular, la proteólisis de los componentes de la matriz extracelular (ECM) y la migración celular. Se produce tanto durante procesos fisiológicos normales como la morfogénesis embrionaria, los procesos de cicatrización de heridas y los procesos de invasión de tejido por parte de células inmunes; como en situaciones patológicas asociada a procesos de tumorogénesis. Debido a su importancia la invasión y migración celular son procesos altamente controlados.

El control de la migración celular, la formación de fibras de estrés y la reorganización del citoesqueleto, están reguladas por una familia de GTPasas (pertenecientes a la superfamilia génica de Ras) que incluye proteínas como Rho (isoformas A, B y C), Rac 1 y 2, Cdc42, Rho D, Rho E, Rho G y TC10 (Ridley 2001 b; Ridley 2001a). Como otras GTPasas, las proteínas de la familia de Rho presentan dos conformaciones alternativas: la activa, en la que la proteína se encuentra unida a GTP y la inactiva en la que lo hace a GDP. Existen además toda una serie de proteínas accesorias que regulan el paso de una conformación a otra regulando así la activación de las GTPasas (Scita 2000).

Los factores intercambiadores de nucleótidos de guanidina (GEFs), las proteínas activadoras de las GTPasas (GAPs) y los inhibidores de la disociación de GDP (GDIs) regulan a estas enzimas (Dransart 2005) .

Los GEFs promueven el intercambio de nucleótidos GDP por GTP, lo que induce una activación de las proteínas de la familia Rho, mientras que las GAPs, que presentan actividad GTPasa intrínseca, promueven su inactivación. Por otra parte los GDIs permiten la estabilización de la conformación inactiva de la proteína impidiendo el intercambio de GDP.

Una vez activadas, las GTPasas interaccionan con sus efectores específicos lo que desencadena una amplia gama de respuestas celulares entre las que se incluyen la reorganización del citoesqueleto de actina y cambios en la transcripción génica. Por lo general, está establecido que Rho está implicada en la formación de fibras de estrés y contactos focales, en la contracción de la miosina y en la polimerización de actina. Rac es, sin embargo, esencial para la formación del lamelipodio (extensión citoplasmática aplanada en la periferia celular relacionada con la migración celular) en el frente de avance de la célula (Small 2002), mientras que Cdc42 está implicada en la formación del filopodio (extensión citoplasmática digitiforme de reconocimiento del medio extracelular).

Los efectores de estas GTPasas están también implicados en la regulación de la migración celular como la quinasa de Rho (ROCK, ROK).

Rock es una serina/treonina quinasa que fue descubierta por primera vez como efector de Rho A y asociada a la formación de fibras de estrés (Leung 1996). Además, Rock está implicada en la fosforilación de MLC induciendo la contracción celular (Niggli 2006).

Se ha establecido que, en células neuronales, la perdida del gen supresor de tumores de la neurofibromina (que codifica para una GAP) induce un aumento en la migración celular (Ozawa y Toshihiro Hara 2005) en un mecanismo dependiente de Rock y que, en células de fibrosarcoma HT1080, Rock es esencial para la regulación de la forma y migración celular (Niggli 2006).

Por otro lado los estudios realizados con la línea celular de cáncer de mama Met-1 han demostrado que una microinyección del dominio catalítico de Rock produce un aumento del número de protusiones celulares y un incremento en la migración celular (Bourguignon 1999).

4.1. Modelo de migración celular.

Durante la migración, la célula debe modificar su morfología en un proceso altamente dinámico que implica la reorganización de los elementos del citoesqueleto y la interacción con los componentes de la matriz extracelular. Por lo general se establece que, para células de tipo epitelial, las células siguen un modelo de migración en 5 pasos (Lauffenburger 1996).

1. Formación de una protusión (lamelipodio) en el denominado frente de migración o avance de la célula. Los monómeros de actina polimerizan originando nuevos microfilamentos que se anclan a proteínas adaptadoras. Esto produce una fuerza de tensión en la membrana que empuja a la célula a desplazarse en una dirección determinada (Pollard y Borisy 2003) (Figura 5). Para que la polimerización de la actina globular se produzca, se requiere la formación previa de un complejo WASP/ARP2/3 que incluye proteínas promotoras de la polimerización de actina (ARP 2/3) (Machesky, Reeves et al. 1997) y proteínas adaptadoras (WASP) (Takenawa 2001). La GTPasa Rac es esencial en la regulación de la reorganización citoesquelética durante la formación del lamelipodio (Arthur 2001; Ridley 2001 b; Ridley 2001a; Hall 1992) .

2. Interacción entre la célula y la matriz, adhesión celular y formación de contactos focales. Durante esta fase, se forman contactos entre las integrinas celulares y los ligandos de la matriz extracelular lo que induce el reclutamiento de proteínas adaptadoras y transductoras de señales (que se unen a las integrinas mediante sus dominios citoplasmáticos) (Blystone 2004; Wozniak 2004). Así, los extremos citoplasmáticos de las integrinas interaccionan de forma directa con proteínas como la α-actinina, la talina y la quinasa de adhesiones focales (FAK) (Mitra 2005) , entre otras, que a su vez interaccionan con proteínas adaptadoras que permiten reclutar proteínas de unión a actina (como la paxilina y vinexina) (Laukaitis 2001; Takahashi 2005) y moléculas reguladoras (PI3K, GTPasas de la familia de Rho) a los contactos focales. Este proceso es altamente dinámico y el equilibrio entre la formación/disociación de estos contactos focales constituye un paso clave en la motilidad celular (Parsons 2000; Mitra 2005).

3. Reclutamiento de proteasas a los contactos focales y proteólisis focalizada. Las metaloproteasas (MMPs), una familia de proteasas dependientes de zinc, juegan un papel de relevancia en los procesos de migración celular siendo las encargadas de degradar los componentes de la ECM (colágeno, laminina) de forma direccional y focalizada. Estas proteinasas, que juegan un papel esencial en los procesos de migración celular, están directamente implicadas en alteraciones patogénicas como la formación de tumores y fenómenos de metástasis. Dada su importancia se estudiarán con detenimiento más adelante.

4. Contracción de las moléculas de actomiosina y translocación del cuerpo celular. Las moléculas de miosina II activada se unen a los microfilamentos de actina, pasándose a denominar filamentos de actomiosina, y generan la contracción. Este proceso involucra a ROCK (Niggli 2006) y la MLCK (quinasa de la cadena ligera de la miosina dependiente de calcio-calmodulina). La fuerza contráctil generada por la contracción de la actomiosina es utilizada por la célula para avanzar el cuerpo celular (que incluye el núcleo y el citoplasma) hacia el frente de migración de la célula (Yamazaki, Kurisu et al. 2005).

5. Desanclaje de los contactos focales en la cola celular. En la cola celular se produce un fenómeno de desensamblaje de las adhesiones focales en un proceso que involucra diversos mecanismos. Proteínas como la gelsolina, que se une a los filamentos de actina en su extremo apical (Wiesner 2003; dos Remedios CG 2003), y la cofilina, impiden la polimerización de las fibras de actina (Giganti A 2005) y promueven el recambio de los monómeros de actina. Por otro lado, proteasas como la calpaína degradan los componentes de los contactos focales (Bialkowska 2005; Bhatt 2002) mientras que la quinasa de adhesiones focales (FAK) promueve el desensamblaje de las adhesiones focales (Mitra 2005; Bertolucci 2005; Hamadi 2005) .

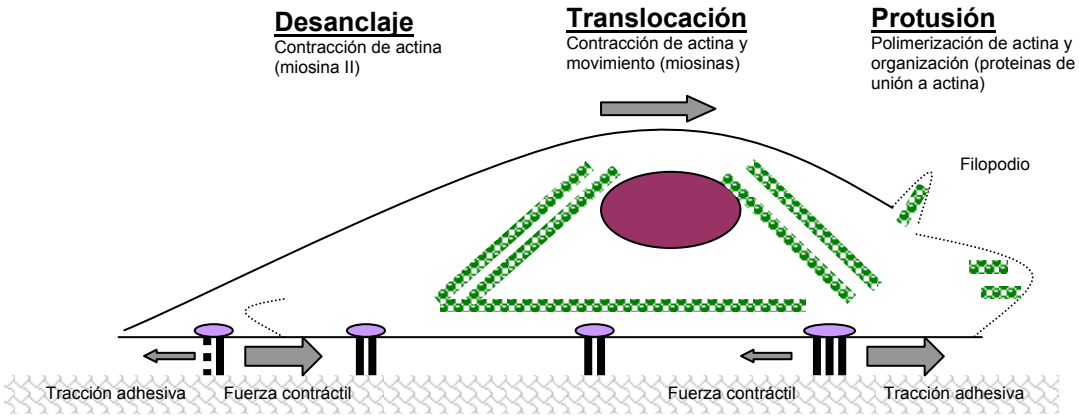

Figura 5. Esquema de migración celular.

5. Metaloproteasas

La degradación de la ECM por medio de proteasas específicas, se considera un requisito previo e indispensable para la migración celular tanto en procesos fisiológicos normales como en situaciones patológicas. Las MMPs forman parte de una familia de 24 miembros constituida por endopeptidasas dependientes de zinc que juegan un papel esencial en la degradación de la ECM durante procesos tan dispares como el desarrollo embrionario, la cicatrización de heridas, la ovulación, la remodelación ósea y el desarrollo normal de la función de células inmunes (macrófagos y neutrófilos). Además, las MMPs están involucradas en fenómenos patogénicos relacionados con una excesiva degradación de la matriz como la artritis reumatoide, la osteoartritis, úlcera gástrica, arteriosclerosis y en fenómenos de formación, crecimiento de tumores y metástasis (Polette, Nawrocki-Raby et al. 2004; Lemaitre V 2006; Mancini and Di Battista 2006) .

Todos los miembros de la familia de las MMPs son sintetizados como pro-enzimas y presentan dos dominios altamente conservados: el llamado pro-dominio y el dominio catalítico. El dominio catalítico contiene un sitio activo que presenta en su secuencia (HEXGHXXGXXH) tres histidinas altamente conservadas. Esta secuencia, permite la unión a los ligandos de zinc necesarios para su activación. [Para revisión consultar (Visse 2003; Borkakoti 2004)].

Dentro de las MMPs podemos distinguir 6 grupos: colagenasas (MMP-1, MMP-8, MMP-13, y MMP-18), gelatinasas (MMP-2 y MMP-9), estromalisinas (MMP-3 y MMP-10), matrilisinas (MMP-7 y MMP-26), metaloproteasas de membrana (que engloban las proteínas trans-membrana tipo I MMP-14, MMP-15, MMP-16 y MMP-24 y las ancladas a glicofosfatidilinositol MMP-17 y MMP-25) y un grupo que engloba a metaloproteasas que no son incluidas en las clasificaciones anteriores (MMP-11, MMP-12, MMP-19 y MMP-20) (Figura 6).

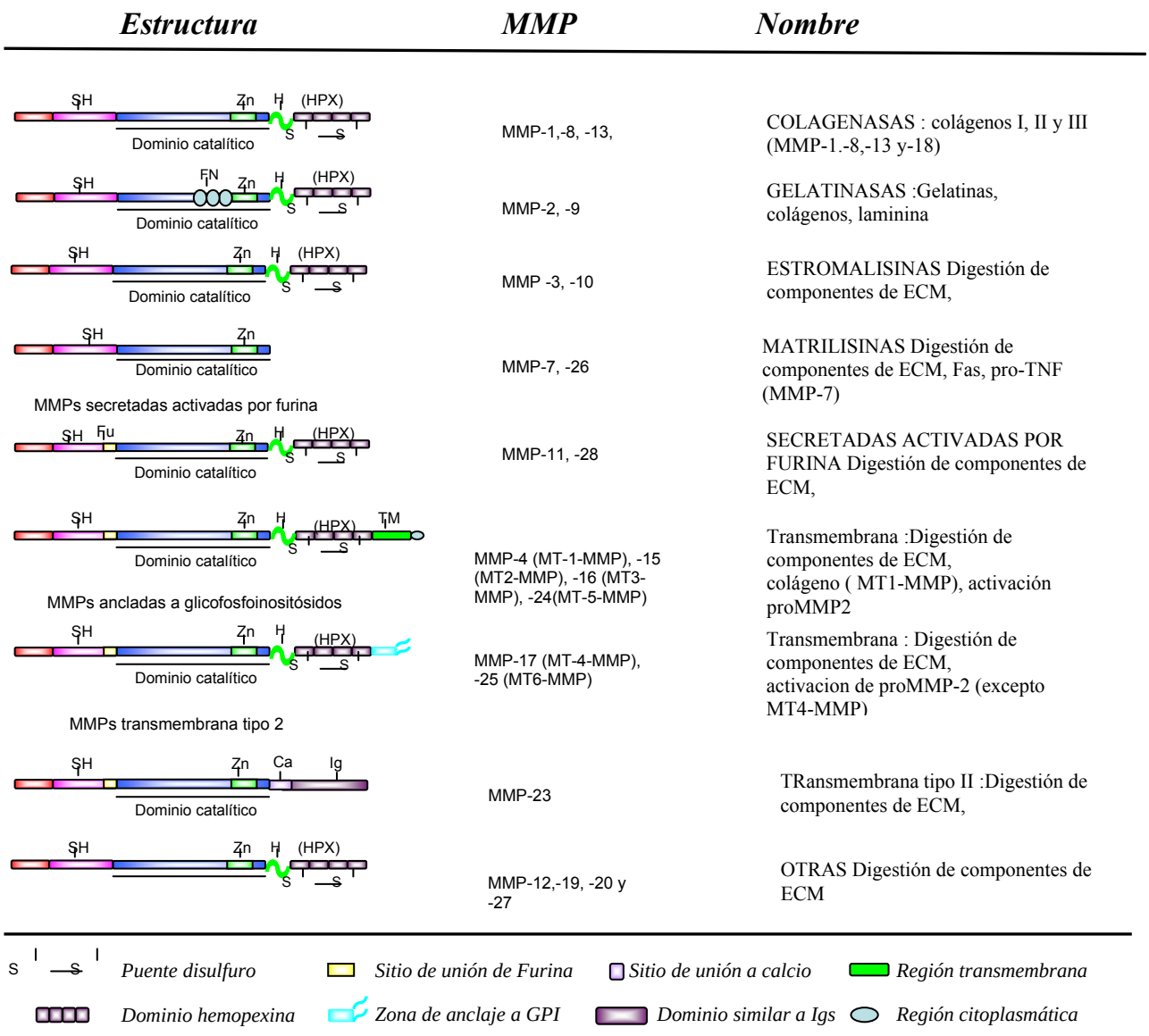

Figura 6. Estructura y función de las MMPs.

Debido a su importancia, las MMPs se encuentran altamente reguladas (a nivel transcripcional, post-transcripcional, de activación y en su localización celular) y poseen inhibidores específicos, los TIMPs (inhibidores tisulares de las metaloproteasas 1-4), que se anclan a la región de unión a ligandos de zinc impidiendo la activación de las metaloproteasas (Jiang Y 2002). Su expresión anómala, al igual que ocurre con las metaloproteasas, está implicada en fenómenos de tumorogénesis (Jiang 2002).

OBJETIVOS

La MAP quinasa quinasa quinasa Cot /tpl-2 se descubrió en un forma truncada/modificada que poseía potencial oncogénico, numerosas evidencias indican que el papel fisiológico de esta quinasa está más relacionado con procesos de inmunidad innata. Cot/tpl-2 está asociada a la formación de tumores por lo que se presenta como una interesante molécula diana para el desarrollo de drogas antitumorales. Aunque se han realizado algunos estudios, por ahora se desconoce el mecanismo de activación de Cot/tpl-2 así como su implicación en las distintas funciones celulares.

Una de las características de las células transformadas es que presentan incrementada su capacidad migratoria, Cot/tpl-2 posee potencial oncogénico y es capaz de inducir, al sobre-expresarse, transformación celular, sin embargo no se ha establecido hasta ahora el posible implicación de Cot/tpl-2 en fenómenos de migración celular.

Los objetivos de esta Tesis Doctoral han sido:

1. Determinar la(s) señal(es) extracelular(es) capaces de inducir una activación de Cot/tpl-2.

2. Estudio del mecanismo de activación de Cot/tpl-2 por estas señales extracelulares y determinación de las moléculas implicadas en el proceso.

3. Determinar la posible existencia de inhibidores específicos de la actividad quinasa de Cot/tpl-2.

4. Determinar la implicación de Cot/tpl-2 en migración celular.

MATERIALES Y MÉTODOS

PUBLICACIÓN 1

El LPS, uno de los componentes mayoritarios de la pared celular de las bacterias Gram-negativas, es un potente activador de macrófagos en los que induce la producción y secreción de citoquinas (como el TNF-α y la IL- 1α) y de metabolitos del ácido araquidónico.

Para poder unirse a su receptor, el LPS interacciona con una proteína soluble del suero denominada LBP (LPS binding protein, proteína de unión al LPS) y, una vez acomplejada con LBP, interacciona con el receptor de superficie CD14 presente en macrófagos. Puesto que CD14 carece de un dominio citosólico que le permita transducir la señal, se requiere una interacción con un receptor adicional perteneciente a la familia de receptores tipo Toll (Toll like receptors, TLR), concretamente el TLR4, que será el encargado de tansducir la señal a través de la membrana.

La estimulación por LPS del TLR4 induce la activación de una ruta de señalización que implica a proteínas como los adaptadores MyD88 y TRAF6 y la quinasa IRAK. Esta ruta permite que se produzca una activación de las rutas de las MAPKs y en particular la vía de las denominadas MAPK clásicas o vía de ERK1/ERK2 mediante un mecanismo que aún no está esclarecido por completo.

El taxol, una potente droga antimitótica, es capaz de mimetizar a determinadas concentraciones, la acción del LPS uniéndose a CD14 y activando el TLR4 en células murinas.

El gen de Cot/tpl-2 codifica para una MAP quinasa quinasa quinasa (MKKK), que es capaz de activar, en estudios de sobreexpresión, las rutas de las MAPK ERK1/ERK2, JNK, p38, y ERK5. En condiciones normales no patológicas Cot/tpl-2 parece activar, de modo más específico y a través de MKK1, a ERK1/ERK2.

En este trabajo se identifican dos señales extracelulares (LPS y Taxol) capaces de incrementar 10 veces la actividad específica de la proteína Cot/tpl-2 endógena siendo esta máxima a los 15 minutos. Además, demostramos que Cot/tpl-2 es la única MKKK que activa MKK1 y por consiguiente la ruta de ERK1/ERK2, en respuesta a estos estímulos.

Por otro lado, nuestros experimentos nos permiten establecer que la disociación de Cot/tpl-2 del complejo estable que forma con la subunidad p105 de NF-κB aunque necesaria, no es causa suficiente para inducir la activación de Cot/tpl-2 y es probable que se produzca otra modificación adicional en Cot/tpl-2.

La utilización de inhibidores la actividad tirosina quinasa como la herbimicina, bloquea la activación de Cot/tpl-2 inducida por LPS o Taxol en macrófagos, lo que indica la implicación de una actividad tirosina quinasa en la activación de Cot/tpl-2.

Nuestros resultados además, han permitido demostrar que el ligando natural del receptor activado por factores proliferadores peroxisomales tipo γ (PPAR-γ), la 15-deoxy-Δ12,14-prostaglandina J_2 (15d-PGJ_2), es capaz de bloquear la activación de Cot/tpl-2 inducida por LPS y la actividad quinasa específica de Cot/tpl-2 *in vitro* mientras que la rosiglitazona (un ligando sintético de PPAR-γ) no produce efecto alguno sobre la activación de Cot/tpl-2. Estos datos nos demuestran que la 15d-PGJ_2 inhibe a Cot/tpl-2 mediante un mecanismo independiente de PPAR-γ.

Al investigar los posibles efectos de la 15d-PGJ_2 sobre la activación de ERK1/ERK2 en respuesta a PMA, pudimos comprobar que la 15d-PGJ_2 no es capaz de de inhibir la activación de MKK1 ni la consecuente activación de ERK1/ERK2 mediada por c-Raf. Así la 15d-PGJ_2 se presenta como un inhibidor específico de Cot/tpl-2.

Considerando los resultados obtenidos proponemos que el efecto antinflamatorio de 15d-PGJ_2 se debe en parte a su capacidad para inhibir la activación de Cot/tpl-2 en respuesta a LPS.

En la citada publicación el doctorando ha contribuido al diseño y realización de los experimentos cuyos resultados se ven plasmados en las figuras 2, 3, 4 y 5. Se puede considerar que su grado de aportación al proyecto es elevado como refleja el hecho de que el doctorando esté considerado como co-autor del manuscrito

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15-Deoxy-Δ12,14-prostaglandin J2 Regulates Endogenous Cot MAPK-Kinase Kinase 1 Activity Induced by Lipopolysaccharide*

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Cot is a MAPK kinase kinase that has been implicated in cellular activation and proliferation. Here, we show that the addition of lipopolysaccharide (LPS) to RAW264 macrophages induces a 10-fold increase of endogenous Cot activity, measured as MAPK kinase kinase 1 activity. Taxol, but not phorbol 12-myristate 13-acetate (PMA), induces a similar activation of Cot. A tyrosine kinase activity is involved in Cot activation by LPS. 15-Deoxy-Δ12,14-prostaglandin J2, but not rosiglitazone, blocks Cot activation by LPS. Furthermore, 15-deoxy-Δ12,14-prostaglandin J2 also inhibited the LPS-induced Cot *in vitro*. However, 15-deoxy-Δ12,14-prostaglandin J2does not inhibit MAPK kinase 1 or ERK1/ERK2 activation/phosphorylation induced by PMA and mediated by c-Raf. Considering these data, we propose that the inhibition of LPS-induced Cot activation is one mechanism by which 15-deoxy-Δ12,14-prostaglandin J2 acts as an anti-inflammatory.

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1 The abbreviations used are: LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; Gö 6850, bisindolylmaleimide I; Herb A, herbimycin A; 15d-PGJ$_2$, 15-deoxy-Δ12,14-prostaglandin J$_2$; PGE$_2$, prostaglandin E$_2$; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase 1; MKKK, MAPK kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PPAR-γ, peroxisome proliferator-activated receptor-γ; TNF-α, tumor necrosis factor α.

INTRODUCTION.

Lipopolysaccharide (LPS)[1] is a major cell wall component of Gram-negative bacteria that potently activates macrophages, inducing the production and secretion of immunoregulators such as TNF-α, interleukin-1α, and arachidonic acid metabolites (for reviews see Refs. 1–3).

Deregulation of the secretion of these pro-inflammatory cytokines is associated with septic shock and other inflammatory conditions such as rheumatoid arthritis. When complexed with a serum protein, LPS binds to the molecule CD14 on the surface of macrophages. The CD14 receptor itself lacks a cytosolic domain, yet it appears to interact with the toll-like receptors that, in turn, can transduce the signal across the plasma membrane (1–4). Indeed, activation of the toll-like receptors triggers an intracellular signaling cascade that involves the adapter proteins MyD 88 and IRAK, and the TRAF6 proteins (for a review see Ref. 5). The LPS signal transduction pathway involves the rapid tyrosine phosphorylation of several proteins and the activation of different mitogen-activated protein kinase (MAPK) cascades, including the classical MAPK pathway (6 –9). However, the precise molecular mechanism by which LPS activates the extracellular signalregulated kinases 1 and 2 (ERK1 and ERK2) in the classic MAPK pathway is still not fully understood.

The Cot/tpl-2 gene encodes a MAPK kinase kinase (MKKK) that in overexpression studies was originally reported to be capable of triggering several MAPK cascades, namely those leading to activation of the MAPKs ERK1/ERK2, c-Jun N-terminal kinase, p38, and ERK5 (10–15). However, more recent studies have shown that peritoneal macrophages from Cot/tpl-2 knockout mice do not activate ERK1/ERK2 in response to LPS, although the activation of c-Jun N-terminal kinase and p38 MAPK pathways remain the same as in wild type mice (15). This suggests that under normal conditions, Cot/tpl-2 may act more specifically within the classical MAPK cascade than when overexpressed in cells. Thus, Cot/tpl-2 is one of the three proteins that can activate MAPK kinase 1 (MKK1) in cells, together with Raf and Mos.

The Cot/tpl-2 proto-oncogene was originally identified in a modified C-terminally truncated form. The disruption of the last coding exon of the human Cot gene or the truncation of its murine homologue, tpl-2, increases its specific activity (16), revealing the oncogenic potential of the gene (16–18). The high levels of Cot expression in malignant human Hodgkin/Reed-Sternberg cells (19) and human breast cancer cells (20) further emphasize this oncogenic potential. The overexpression of Cot in different cells induces the activation of several transcription factors, including activator protein 1 (12, 14, 21), NFAT (22–24), NFkB (13, 23, 25, 26), and E2F (27). The involvement of Cot in the secretion of TNF-a in T-cells and macrophages (15,21) and in the secretion of interleukin-2 in T lymphocytes has also been established (22, 23). Furthermore, Cot plays an essential role in inducing transcription of the gene encoding cyclooxygenase-2 (19, 24).

Thus, Cot is a key component in the activation of T-cells and macrophages. Indeed, signals that activate T-cells increase the levels of Cot mRNA, suggesting that the expression of Cot protein increases during the G0 transition of T lymphocytes (28, 29). In this paper we identify two extracellular signals that increase the specific activity of endogenous Cot, namely LPS and Taxol. Furthermore, we demonstrate that Cot is the only MKKK that activates MKK1, and hence ERK1 and ERK2, in macrophages in response to these signals. We also provide evidence that 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2), but not rosiglitazone, blocks the LPS-induced activation of Cot and that it also inhibits Cot activity in vitro. These data indicate that 15d-PGJ_2 inhibits LPS-induced Cot activation through a peroxisome proliferator-activated receptor-γ (PPAR-γ)-independent mechanism.

EXPERIMENTAL PROCEDURES.

Materials— Fetal bovine serum (FBS) was purchased from Invitrogen. Prostaglandin E2 (PGE2), herbimycin A (Herb A), Gö 6850 (bisindolylmaleimide I), and 15d-PGJ2 were from Calbiochem; rosiglitazone was a generous gift from GlaxoSmithKline; phorbol 12-myristate 13-acetate (PMA), LPS, sodium orthovanadate, LY 294002, and myelin basic protein were from Sigma; complete proteinase inhibitor mixture was from Roche; and [^{35}S]methionine and [^{35}S]cysteine labeling mixture was from Amersham Biosciences. Taxol (paclitaxel) was generously provided by Dr. Eva López (Bristol-Myers Squibb Co.). Antiphospho-ERK1/ERK2 and anti-ERK2 antibodies were purchased from Zymed Laboratories Inc. Laboratories and anti-phospho-MKK1 plus anti-MKK1 antibodies from Cell Signaling Technology. Antibodies used to immunoprecipitate Cot and c-Raf were raised in sheep against theCot immunogenic peptide, CQSLDSALFDRKRLLSRKELE, and bacterially expressed c-Raf protein by the Division of Signal Transduction Therapy at the University of Dundee (performed in P. Cohen´s laboratory).The rabbit anti-Cot antibody utilized for Western blot analysis has been described previously (22).

Cell Culture, Metabolic Labeling, and Stimulation—RAW264 macrophages were maintained in a 95% air, 5% CO2 atmosphere in RPMI plus 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin, 100 μg/mlstreptomycin. One day prior to stimulation, macrophages were plated at a density of 1.2 x 10^6 cells/90-cm plate. Three hours later, themedium was removed and replaced

overnight with 8 ml of RPMI with 1% (v/v) heat-inactivated FBS. For metabolic labeling, cells were kept inmethionine-, cysteine- and glutamine-free RPMI for 20 min and then pulsed overnight with RPMI plus 1% heat-inactivated FBS dialyzed against phosphate saline buffer plus 100 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine labeling mixture. Unless otherwise indicated, cells were stimulated with 500 ng/ml LPS, 50 μM Taxol, or 0.4 μg/ml PMA for the times specified in the figure legends. Where indicated, 15d-PGJ_2 (10μM), rosiglitazone (10μM), or PGE_2 (5 mM) was added to the medium 60 min before stimulation. Go¨6850 (1μM) and LY 294002 (50 μM) were added to the medium 30 min before stimulation and Herb A (1 μg/ml) 3 h before stimulation.

Immunoprecipitation and Assay of Cot and c-Raf—After stimulation, the medium was removed, and the cells were solubilized in 0.5 ml of ice-cold lysis buffer (50 mM Tris acetate (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, and complete proteinase inhibitor mixture, 1 tablet/10 ml). The samples were then frozen in liquid nitrogen and stored in aliquots at -70 °C until analysis. Cell extracts were thawed at 0 °C and centrifuged for 10 min at 24,000 x g, and protein concentration was determined by the Bradford method. The protein c-Raf was immunoprecipitated for 1 h at 4 °C by incubating 0.5 mg of cell lysate protein with 1 _g of c-Raf antibody that had been coupled to protein G-Sepharose. Cot was immunoprecipitated for 3 h at 4 °C by incubating 0.5 mg of cell lysate protein with 1.5 μg of Cot antibody covalently bound to protein G-Sepharose. The different immunoprecipitates were washed and subjected to a two-step kinase assay as described previously for c-Raf (30). One unit of Cot or c-Raf activity was that amount of enzyme that incorporated 1 nmol of phosphate into myelin basic protein in 1 min.

Western Blot Analysis—Western blotting was performed by resolving 35 μg of cell lysate protein on 10% SDS-polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membranes and probed with anti-phospho-ERK1/ERK2 or anti-phospho-MKK1 antibodies. The blots were developed using a chemiluminescent method (ECL, Amersham Biosciences). As a control of protein loading on the gels, the polyvinylidene difluoride membranes were re-probed with anti-ERK2 or anti-MKK1 antibodies.

RESULTS.

LPS and Taxol Activate Cot but Not c-Raf—Treatment of RAW264 macrophages with LPS (500 ng/ml) produced a time-dependent activation of endogenous Cot activity (Fig. 1). Maximal activation reached about 10-fold the basal levels and was attained 15 min after the addition of LPS. In contrast, LPS stimulation did not augment the activity of c-Raf. Because Taxol is reported to mimic LPS-induced activation of murine macrophages through an interaction with CD14 (31–33), we also examined the effect of stimulating RAW264.7 macrophages with 50 μM Taxol for different time periods on Cot and c-Raf activity. Not only did Taxol increase the specific activity of endogenous Cot, although to a lesser extent than LPS, but also Taxol failed to up-regulate c-Raf activity (Fig. 1).

PMA Activates c-Raf but Not Cot—It has been well established that through protein kinase C (PKC), the tumor-promoting phorbol esters can trigger the activation of the classical MAPK cascade in a wide variety of cells, including macrophages. We therefore set out to compare the mechanism by which PMA and LPS activate this signaling pathway in macrophages. Stimulation of RAW264 cells with 0.4 μg/ml PMA increased c-Raf activity about 10-fold, with the maximal increase occurring 5 min after stimulation. However, in sharp contrast to LPS or Taxol, PMA did not activate Cot activity (Fig. 1), indicating that PMA and LPS/Taxol activate the MAPK cascade in different ways.

The Mechanism by Which LPS Activates Cot—In Western blots of endogenous immunoprecipitated Cot, a single band that migrated in the position expected for the Cot polypeptide was observed (Fig. 2A). On this basis, we assessed the possibility that LPS increased Cot activity by modulating its association/dissociation with putative regulatory subunit(s). Unstimulatedand LPS-stimulated cells were labeled with [^{35}S]methionine and [^{35}S]cysteine, and endogenous Cot was immunoprecipitated from the cell lysates. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and subsequent autoradiography revealed a single radiolabeled band of the expected size for Cot (Fig. 2B). The absence of any other labeled protein implied that Cot activation by LPS does not result from its association with, or dissociation from, any other protein. Another possible mechanism for Cot activation might

involve its phosphorylation at one or more residues. However, treatment of Cot immunoprecipitated from LPS-stimulated cells with high concentrations of the catalytic subunit

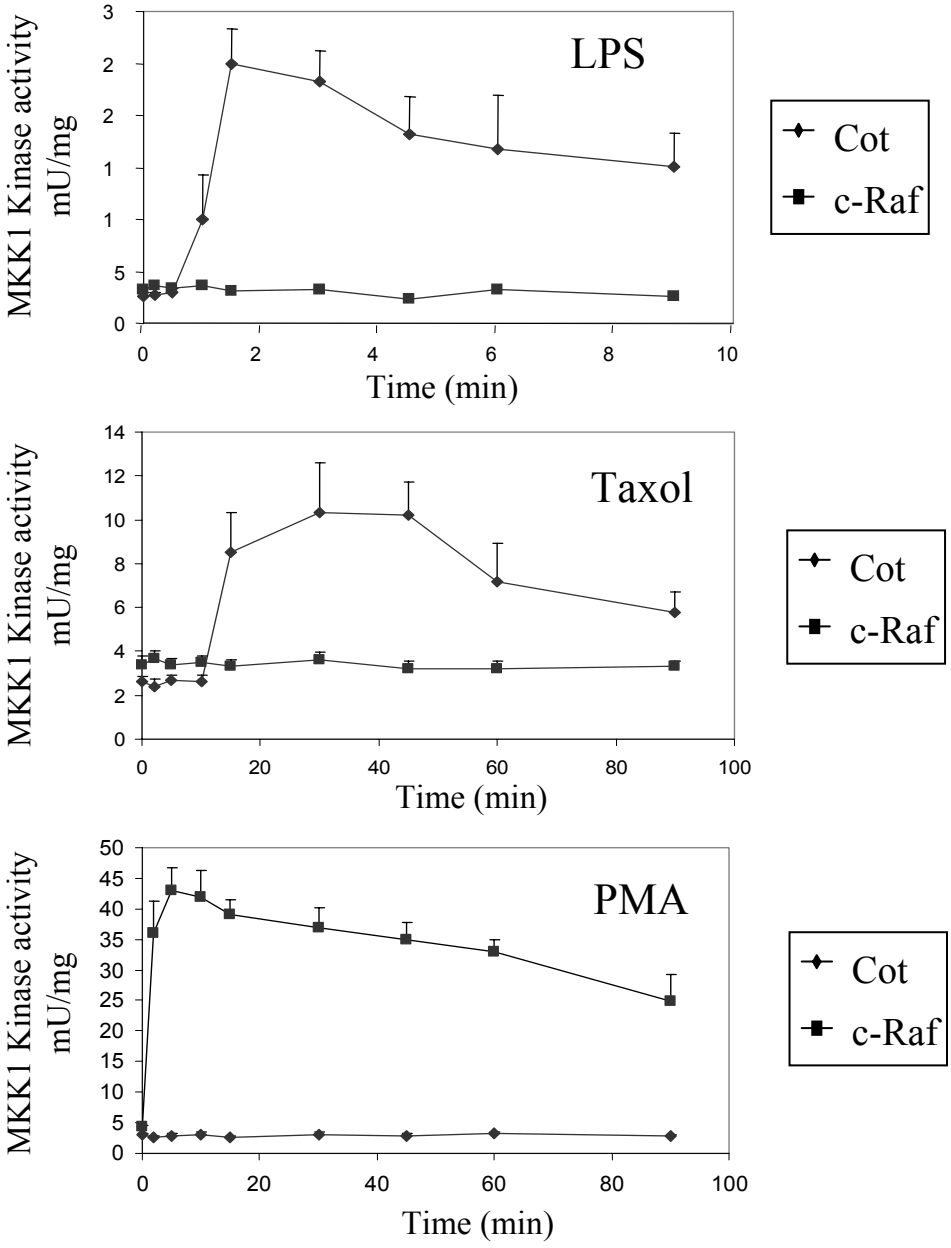

FIG. 1. Activation of MKK1 kinase activity by LPS and PMA in RAW264 macrophages. Macrophages were stimulated with LPS (500 ng/ml), Taxol (50 mM), or PMA (0.4mg/ml) for the indicated times. Cot and c-Raf were immunoprecipitated from lysates and assayed for protein kinase activity as described under"Experimental Procedures." The results are represented as the mean ± S.E. for three determinations from three separate dishes.

of protein phosphatases 1 or 2A or protein tyrosine phosphatase 1B did not affect the activity of Cot (data not shown). Moreover, the inhibition of serine/threonine phosphatases PP1 or PP2A, potential regulators of Cot phosphorylation, by incubating RAW cells with okadaic acid, did not increase Cot activity (data not shown). Finally, we also transfected Cot into HEK293 cells, which were then stimulated with the protein tyrosine phosphatise inhibitor orthovanadate, or with orthovanadate plus okadaic acid. However, neither of these treatments increased the activity of Cot (data not shown).

Taken together, these data indicate that it is unlikely that serine/threonine phosphorylation of Cot is responsible for the way in which LPS influences its level of activity.

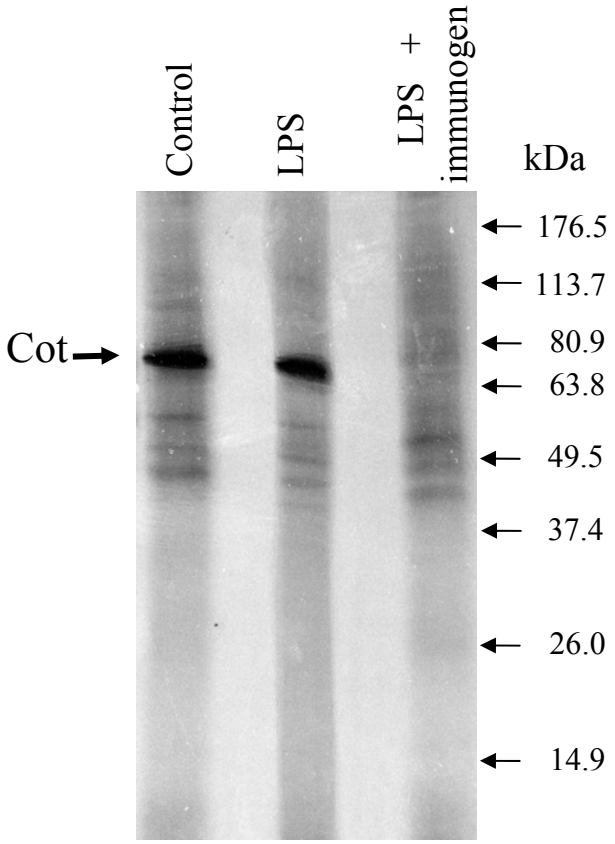

FIG. 2. **Immunoprecipitation of endogenous Cot from RAW264 macrophages stimulated or not with LPS.** *A*, Cot immunoprecipitated from control cell lysates (250 mg) was subjected to Western blot analysis with rabbit anti-Cot antibody. *B*, 35S-labeled RAW cells were stimulated or not stimulated with 500 ng/ml LPS for 15 min. Cot was immunoprecipitated from the different RAW cells extracts in the presence or absence of 10 mM immunogenic peptide and resolved by SDS-polyacrylamide gel electrophoresis followed by autoradiography and radioactivity quantification by Instant Imager. The figure shows one of three experiments performed.

Inhibitors of Protein Tyrosine Kinases Block the Activation of Cot by LPS—Herb A is an irreversible inhibitor of protein tyrosine kinases, and it has been found to block both LPS-stimulated tyrosine phosphorylation and the activation of ERK1/ERK2 in macrophages (6).

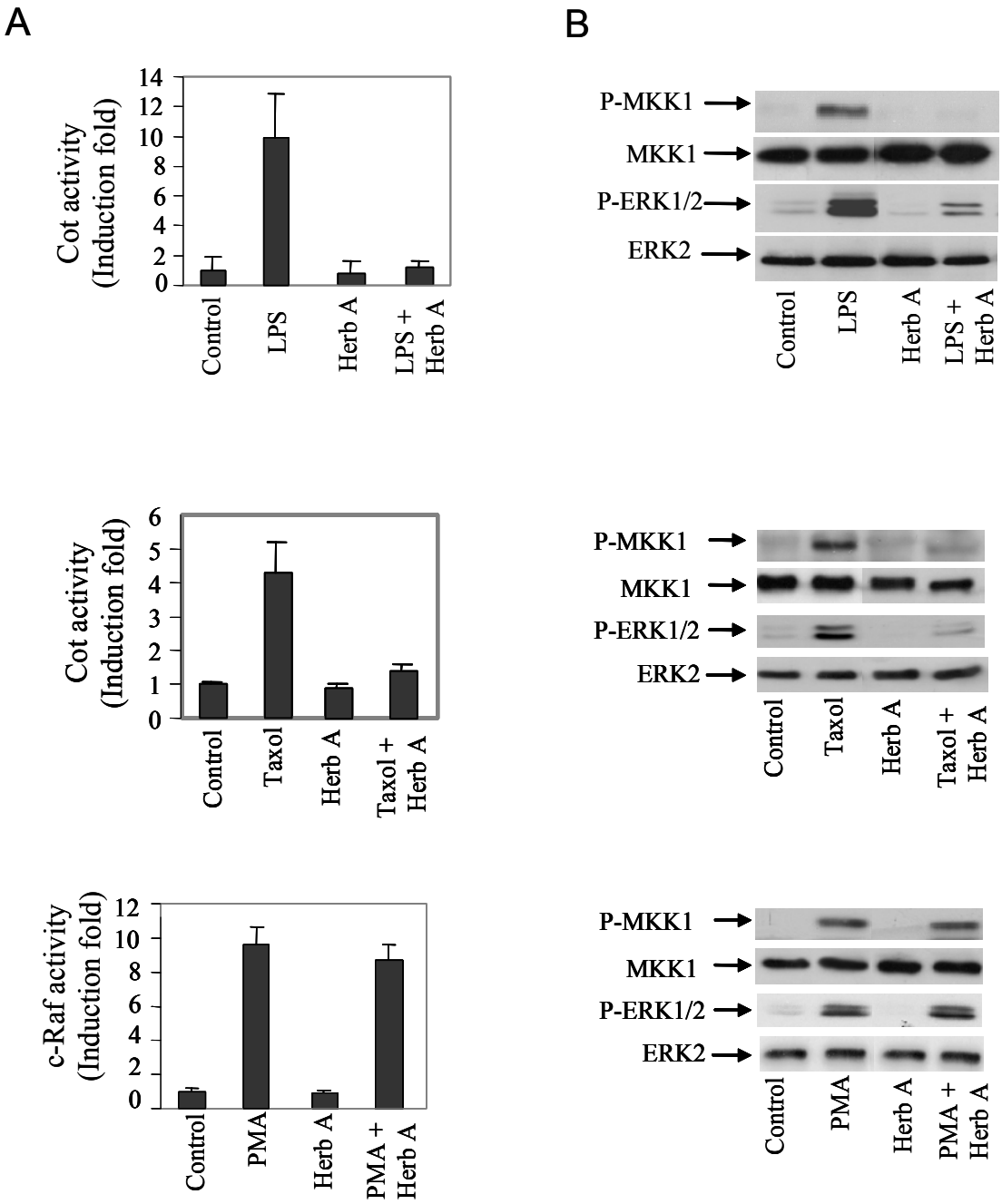

FIG. 3. **Herb A inhibits Cot activation by LPS.** Macrophages were preincubated with Herb A (1 mg/ml) for 3 h and then stimulated with LPS (200 ng/ml) for 15 min, with Taxol (50 mM) for 20 min, or with PMA (0.4 mg/ml) for 5 min. *A*, Cot or c-Raf was immunoprecipitated from different LPS-, Taxol-, or PMA-treated cells, and MKK1 kinase activity was measured. The results are represented as the mean ± S.E. for three different determinations from two separate dishes. The value 1 corresponds to Cot or c-Raf activities from control cells. *B*, lysates of the differently treated cells were electrophoresed and immunoblotted with anti-phospho ERK1/ERK2 and anti-phospho-MKK1 antibodies. As a control of protein loaded, total ERK2 and total MKK 1 levels, respectively, were tested. Similar results were obtained in three different experiments.

Therefore, we analyzed the effects that this inhibitor might have on Cot activation.Preincubating RAW264 cells with Herb A prevented the activation of Cot by LPS or Taxol (Fig. 3A), although the same treatment did not affect the activation of c-Raf by PMA. Furthermore, Herb A inhibited the activation of MKK1 and ERK1/ERK2 by LPS or Taxol but not by PMA (Fig. 3B). Similar results were also obtained when Herb A was replaced by genistein or tyrphostin AG825, other protein tyrosine kinase inhibitors not structurally related to Herb A (data not shown).

The Activation of Cot by LPS Is Independent of Phosphatidylinositol 3-Kinase and Protein Kinase C—It has been reported that protein kinase B, which is activated "downstream" of phosphatidylinositol 3-kinase (PI3K), induces the phosphorylation of the C terminus of Cot and that this event is accompanied by an increase in NF-κB activation (26). However, the preincubation of RAW264 cells for 30 min with an inhibitor of PI3K, LY 294002 (50 μM), did not block the phosphorylation of ERK1/ERK2 or MKK1 upon stimulation with LPS or PMA (data not shown). Moreover, co-transfection of an active form of PI3K together with Cot in HEK293 cells did not increase the activity of Cot, whether or not the cells were exposed to orthovanadate (data not shown). Protein kinase Cε (PKCε) has been reported to play a critical role in macrophage activation and in the generation of TNF-α (34). Because Cot also participates in TNF-α secretion (15, 21), we decided to evaluate the role that PKCε plays in regulating activation of the classic MAPK cascade via LPS and PMA. As expected, incubation of RAW cells with Gö 6850, an inhibitor of PKCα, PKCβ1, PKCδ, and PKCε, blocked the PMA-induced activation of c-Raf, MKK1, and ERK1/ERK2. However, preincubation with Gö 6850 did not affect the LPS-induced activation of Cot or the phosphorylation of MKK1 and ERK1/ERK2 (Fig. 4), indicating that neither PI3K nor PKCε is involved in the activation of Cot by LPS.

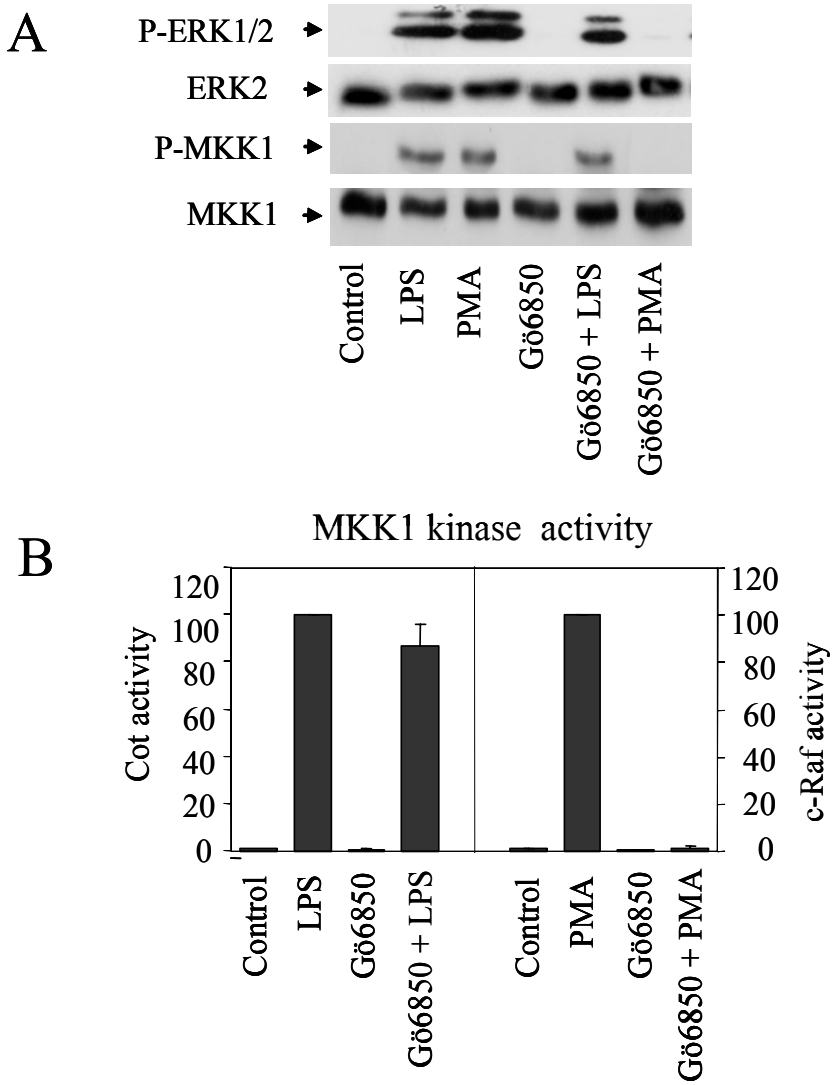

FIG. 4. Gö6850 does not inhibit Cot activation by LPS. Macrophages were preincubated with Gö6850 (1 μM) for 30 min and then stimulated with LPS (200 ng/ml) for 15 min or with PMA (0.4μg/ml) for 5 min. The phosphorylation states of ERK1/ERK2 and MKK1 (*A*) and Cot or c-Raf activities (*B*) were measured as described under "Experimental Procedures." The value 100% corresponds to Cot or c-Raf activities when cells were stimulated, respectively, with LPS or PMA.The figure shows one of three experiments performed, and the graph represents the mean of three different determinations ± S.E.

15d-PGJ2 Blocks the Activation of Cot by LPS—Ligands of PPAR-γ are negative regulators of macrophage activation (35–39). Preincubation of RAW264 macrophages with 15d-PGJ_2 (10μM), a potent natural ligand of PPAR-γ, completely suppressed the activation of Cot by LPS or Taxol (Fig. 5A). Consistent with this finding, when cells were preincubated with 15d-PGJ_2, neither MKK1 nor ERK1/ERK2 were activated by LPS. Nevertheless preincubation with 15d-PGJ2 did not decrease the levels of LPS-induced tyrosine phosphorylation as determined by Western blot analysis (data not shown). On the other hand, neither the PMA-induced activation of c-Raf nor the PMA induced phosphorylation of MKK1 and ERK1/ERK2 was affected by preincubation with 15d-PGJ_2 (Fig. 5A). To address the specificity of 15d-PGJ_2, RAW macrophages were also pre-incubated with 5mM PGE_2 prior to stimulation with LPS. Exposure to PGE_2 did not inhibit the LPS-induced activation of Cot (Fig. 5B). Furthermore, we analyzed the effect of rosiglitazone, a synthetic PPAR-γ ligand with an affinity that is equal to or exceeds the affinity of 15d-PGJ_2 (40), on Cot activation by LPS stimulation. In contrast to 15d-PGJ_2, rosiglitazone does not contain the cyclopentenone ring with the α,β- unsaturated carbonyl group that has been proposed to produce a biological activity independent of PPAR-γ (40). Preincubation of RAW264 cells with rosiglitazone (10 μM) did not inhibit LPS-induced activation of Cot (Fig. 5B) suggesting that 15d- PGJ_2 acts through a mechanism independent of PPAR-γ. To examine the effect of 15d-PGJ_2 on Cot activity in vitro, cells were stimulated with LPS, and the activity of Cot immunoprecipitated from these cell lysates was measured in the presence or absence of 15d-PGJ_2 (10 μM). At this concentration of 15d-PGJ2, Cot activity was inhibited by about 75%. The same concentration of rosiglitazone did not inhibit Cot activity in vitro (data not shown). On the other hand, and in contrast to the inhibition of Cot activity by 15d-PGJ_2, only a small reduction in PMA-stimulated c-Raf activity was observed in the presence of 15d-PGJ_2 in the in vitro assay (Fig. 5C).

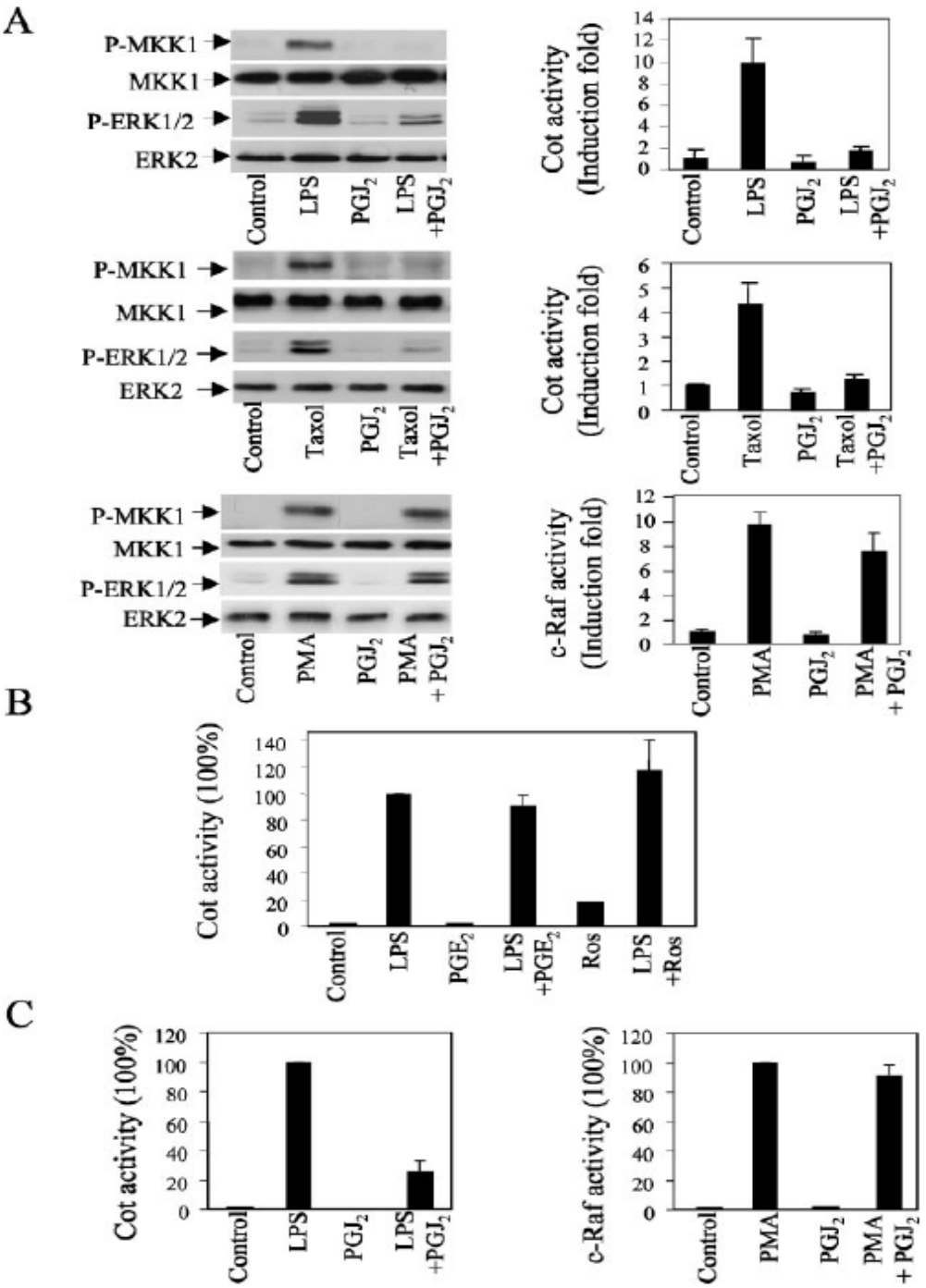

FIG. 5. **15d-PGJ2 inhibits ERK1/ ERK2 activation via Cot in RAW macrophages.** *A*, RAW264 macrophages were preincubated with 15d-PGJ_2 (*PGJ_2*, 10 μM) for 60 min and then stimulated with LPS for 15 min (200 ng/ml), with Taxol (50 μM) for 20 min, or with PMA for 5 min (0.4 μg/ml), and the phosphorylation state of ERK1/ERK2 and MKK1, as well as Cot or c-Raf activities, was determined. The figure shows one of three experiments performed, and the graphs represent the mean of three different determinations ± S.E. The value 1 corresponds to Cot or c-Raf activities from control cells. *B*, RAW264 macrophages were preincubated with PGE_2 (5 mM) or rosiglitazone (*Ros*, 10 μM) for 60 min and then stimulated with LPS (200 ng/ml) for 15 min, and Cot activity was determined. The graph represents the mean of three different determinations ± S.E. The value 100% correspond to Cot activity from LPS-activated cell lysates. *C*, Cot or c-Raf was immunoprecipitated from macrophages stimulated, respectively, with LPS or PMA. The MKK1 kinase activity of the different MKKKs was measured in the presence or absence of 10 μM 15d-PGJ_2. The results are represented as the mean± S.E. for two different determinations from three separate dishes. The value 100% corresponds to Cot or c-Raf activities assayed in the absence of 15d-PGJ_2.

DISCUSSION.

In this paper we have demonstrated that the activation of macrophages with LPS triggers a 10-fold stimulation of endogenous MKK1 kinase activity of Cot. This stimulation reaches a maximum after 15 min and is correlated with the time of activation of ERK1/ERK2 observed both here and in previous studies (6, 41, 42). Our data exclude the possibility that c-Raf connects macrophage stimulation by LPS to the activation of ERK1/ERK2 as has been previously proposed (43–51). Nevertheless, they are consistent with the report that LPS stimulation does not activate ERK1/ERK2 in peritoneal macrophages derived from *Cot/tpl-2* knockout mice (15). Thus, Cot appearsto be the LPS-stimulated MKKK that activates the classical MAPK cascade in macrophages. LPS-induced activation of Cot/tpl-2 does not appear to be triggered by the phosphorylation of the Cot/tpl-2 peptide itself, because treatment with high concentrations of protein phosphatise 2A, protein phosphatase 1, or protein tyrosine phosphatise 1B did not inactivate Cot/tpl-2 immunoprecipitated from LPS-stimulated macrophage extracts. A 47-amino acid deletion of the Cot/tpl-2 C terminus increases its activity (16,18), and it has been shown that Cot mRNA levels are increased by activating stimuli in T-cells (28, 29). However, Cot pull-down experiments indicate that neither of these mechanisms can be responsible for the activation of Cot by LPS. However, the stabilization of Cot mRNA levels after 2 h of LPS stimulation in RAW macrophages[2] may involve a slower and secondary mechanism for enhancing Cot/tp1–2 activity (data not shown). It has been reported recently that in unstimulated cells, Cot/tpl-2 is present in a complex with p105, the precursor of the p50 subunit of the NFκB transcription factor (52, 53), and that LPS induces the dissociation of Cot from p105 (52). We may have been unable to detect p105 in Cot immunoprecipitates from unstimulated cells because the antibody we used was raised against a synthetic peptide corresponding to a sequence near the C terminus of Cot/tpl-2, which is reported to be near the binding site for p105 (53). Therefore, our antibody may dissociate p105 from Cot or may only recognize unbound Cot. Taking into account these recent findings, LPS-induced activation of Cot may require the dissociation of p105 from Cot, perhaps involving the phosphorylation of p105 or another modification of either p105 or Cot. However, because our antibody does not itself induce the activation of Cot in unstimulated cells, the dissociation of p105 from Cot may be only one of the events required for Cot activation.

[2] **M. Caivano, C. Rodriguez, P. Cohen, and S. Alemany, unpublished results**.

The overexpression of Cot with protein kinase B in HEK293 cells has been reported to trigger the phosphorylation of two serine residues at the C terminus of Cot (26). Protein kinase B is activated "downstream" of PI3K; however, PI3K does not appear to be involved in the activation of Cot following LPS stimulation, and hence the induction of the classic MAPK cascade. Indeed, LY294002, an inhibitor of PI3K, does not prevent Cot activation at concentrations that inhibit the activation of protein kinase B. In addition, after LPS stimulation, serine/ threonine-specific (PP1, PP2A) and tyrosine-specific (PTP-1B) phosphatases did not affect the activity of Cot *in vitro*, nor were inhibitors of serine/threonine-specific (okadaic acid) or tyrosine-specific (orthovanadate) phosphatases capable of activating Cot. On the other hand, we found that incubation of macrophages with compounds reported to inhibit protein tyrosine kinases also inhibited Cot activation by LPS or Taxol but not by PMA. This again indicates that in macrophages, different stimuli activate the classical MAPK cascade, using distinct MKKKs. This hypothesis is reinforced by the fact that 15d-PGJ_2 blocks the activation of Cot/tpl-2 and ERK1/ERK2 by LPS or Taxol but not by PMA, and further, by the finding that Gö6850 blocks the activation of Cot/tp1–2 and ERK1/ERK2 by PMA but not by LPS.

Prostaglandin 15d-PGJ2 is a natural ligand of PPAR-γ, a family of nuclear receptors that functions in ligand-activated transcription (54). PPAR-γ ligands have recently been implicated in controlling inflammation and particularly in modulating proinflammatory cytokine production. Indeed, some PPAR-γ ligands inhibit the production of TNF-α, interleukin-1α, interleukin-6, nitric-oxide synthase in monocytes, activated macrophages, or endothelial cells (35–38, 55). In this context, itis noteworthy that the effects of the different PPAR-γ ligands on inflammation do not always correlate with their ability to activate PPAR-γ. Thus, it has been suggested that some ligands, such as 15d-PGJ_2, can also act though a mechanism independent of PPAR-γ (36, 37) and other nuclear receptors.This PPAR-γ-independent activity requires micromolar concentrations of 15d-PGJ_2 (37, 38), and it has been proposed that the chemically reactive α,β-unsaturated carbonyl moiety of the cyclopentenone ring alkylates exposed cysteine residues on target proteins, thereby modifying their function (37, 40). The fact that 15d-PGJ_2 suppresses both LPS-induced Cot activity in intact cells as well as *in vitro* indicates that this activity is independent of PPAR-γ. Indeed, rosiglitazone, which does not contain the cyclopentenone ring with the α,β -unsaturated carbonyl moiety, cannot mimic 15d-PGJ_2-mediated inhibition of LPS-induced Cot activation. To our knowledge, the concentration of 15d-PGJ_2 in serum has never been measured, and it is therefore unclear whether

it may accumulate to the concentrations capable of inhibiting Cot *in vivo*. Indeed, after the induction of an inflammatory response by injecting carrageenin into rats, the concentration of 15d-PGJ2 measured in rat inflammatory exudates was reported to be 3 nM (56), far below that needed to inhibit the activation of Cot in our experiments. Therefore, further work is needed to establish whether the effect of 15d-PGJ_2 on Cot is physiologically or pharmacology relevant. Nevertheless, if the anti-inflammatory effects of 15d-PGJ_2 are indeed mediated by its effects on Cot, this would reinforce the potential importance of Cot as a drug target.

In summary, the results presented in this paper together with those obtained from Cot "knockout" mice (15) indicate that Cot plays a key role in the LPS-induced activation of the classical MAPK cascade and in the subsequent production of a number of inflammatory cytokines and inflammatory mediators in macrophages. Thus, Cot is potentially an attractive target for the development of improved anti-inflammatory drugs, because its inhibition would not affect the activation of the classic MAPK cascade by growth factors and other agonists known to activate this pathway in many cells and tissues via the activation of one or more isoforms of Raf.

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PUBLICACIÓN 2

Los procesos inflamatorios incrementan los niveles intracelulares de interleuquina 1 (IL-1), lo que induce un amplio número de respuestas biológicas. El receptor específico para esta citoquina (el receptor de IL-1) pertenece a la superfamilia génica de los receptores de IL-1 y a los receptores tipo Toll (IL-1R/TLR) que se caracterizan por la presencia de un dominio, en su región citoplasmática, que es requerido para la transducción de la señal y reclutamiento de proteínas adaptadoras. Este dominio es denominado TIR o dominio del receptor de Toll/IL-1.

Aunque los distintos IL-1R/TLR presentan distinta especificidad por las distintas moléculas adaptadoras, el adaptador más común de estos receptores es la proteína MyD88.

MyD88 es capaz de interaccionar con el receptor de IL-1 o el receptor TLR a través de su dominio TIR y, gracias a su dominio de muerte, reclutar a la serina treonina quinasa IRAK. Una vez IRAK es incorporada al complejo, es fosforilada y activada en respuesta a IL-1. Esta fosforilación permite que IRAK sea capaz de reclutar a TRAF6. A continuación, y en una acción conjunta con una enzima conjugadora de ubiquitina, el heterodimero TRAF6/enzima cataliza la adición de una cadena de poliubiquitina a la quinasa TAK1 y a la propia TRAF6. Una vez activada, TAK1 se une varias proteínas denominadas TAB y activa por fosforilación a diversas MKKs que a su vez, regulan la activación de las rutas de señalización de p38 y JNK. Además TAK induce una activación de las proteínas del complejo IKK, lo que permite la degradación del inhibidor de NFκB (IκBα).

El proto-oncogen humano Cot fue identificado en una forma truncada/modificada en su extremo carboxilo terminal. La disrupción del último exón codificante del gen humano o la truncación de su homólogo murino tpl-2 induce transformación celular. El gen Cot/tpl-2 codifica para una MAP quinasa, quinasa, quinasa (MKKK) que al sobreexpresarse produce la activación de distintas rutas de MAP quinasas como: ERK1/ERK2, JNK, p38γ y ERK5

La forma salvaje de la proteína Cot/tpl-2 presenta una vida media corta, mientras que las formas truncadas, carentes del extremo carboxilo terminal, presentan una actividad específica y vida media incrementada con respecto a la forma salvaje lo que explica la capacidad transformante de la proteína truncada.

Estudios previos han permitido establecer que, en macrófagos, Cot/tpl-2 es la única MKKK que activa la ruta de ERK1/ERK2 en respuesta a LPS. Otros estímulos como la estimulación por cadenas de ADN-CpG-del TLR9 y la activación de los receptores de TNFα o CD40L en células B, son también capaces de inducir una activación de Cot/tpl-2.

Estudios recientes han demostrado que en macrófagos no activados, la proteína Cot/tpl-2 forma un complejo estable e inactivo con la subunidad p105 de la proteína NFκB. Al estimular con LPS, se produce una activación de la quinasa IKK cuya subunidad β fosforila p105 NFκB que es degradado rápidamente por el proteasoma a p50 NFκB. La degradación de NFκB produce la liberación de Cot/tpl-2 permitiéndole así activar MKK1 y, por consiguiente, ERK1/ERK2. A continuación Cot/tpl-2 es también degradada por el proteasoma.

En este trabajo demostramos que Cot/tpl-2 es la única MKKK implicada en la activación de ERK1/ERK2 en respuesta a IL-1 y que la actividad quinasa de esta proteína es imprescindible para la inducción de los niveles de mRNA de las quimocinas IL-8 y MIP1-β en respuesta a IL-1.

La estimulación de los receptores de IL-1/TLR por sus ligandos específicos induce la activación de la ruta de NFκB en un mecanismo que implica la actuación de diversas moléculas adaptadoras entre las que se encuentran MyD88 y TRAF6. Nuestros experimentos han demostrado que la activación de Cot/tpl-2 en respuesta a IL-1 y la subsecuente activación de la ruta de ERK1/ERK2 es dependiente de TRAF6 e IKKβ lo que nos permite establecer una relación directa entre la activación de Cot/tpl-2 y la ruta de NFκB.

Además, el PP1 (inhibidor selectivo de la familia de las Src tirosinas quinasas) inhibe la activación de ERK1/ERK2 en respuesta a IL-1 pero no bloquea la disociación ni la consecuente degradación de Cot/tpl-2, indicando que al menos dos rutas de señalización distintas están involucradas en el mecanismo de activación de Cot/tpl-2.

El doctorando ha contribuido a la realización y diseño de los experimentos en concreto en los mostrados en las figuras 1, 2, 3 y 4 constando como co-autor del manuscrito.

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TRAF6 and Src kinase activity regulates Cot activation by IL-1.

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Cot is one of the MAP kinase kinase kinases that regulates the ERK1/ERK2 pathway under physiological conditions. Cot is activated by LPS, by inducing its dissociation from the inactive p105 NFκB-Cot complex in macrophages. Here, we show that IL-1 promotes a 10-fold increase in endogenous Cot activity and that Cot is the only MAP kinase kinase kinase that activates ERK1/ERK2 in response to this cytokine. Moreover, in cells where the expression of Cot is blocked, IL-1 fails to induce an increase in IL-8 and MIP-1βmRNA levels. The activation of Cot-MKK1-ERK1/ERK2 signalling pathway by IL-1 is dependent on the activity of the transducer protein TRAF6. Most important, IL-1-induced ERK1/ ERK2 activation is inhibited by PP1, a known inhibitor of Src tyrosine kinases, but this tyrosine kinase activity is not required for IL-1 to activate other MAP kinases such as p38 and JNK. This Src kinases inhibitor does not block the dissociation and subsequently degradation of Cot in response to IL-1, indicating that other events besides Cot dissociation are required to activate Cot. All these data highlight the specific requirements for activation of the Cot-MKK1-ERK1/ERK2 pathway and provide evidence that Cot controls the functions of IL-1 that are mediated by ERK1/ERK2.

Keywords: IL-1; Cot/tpl-2; ERK1/ERK2; IL-8; MIP-1β; TRAF6 Src tyrosine kinases

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Abbreviations: HerbA, herbimycin A; IL, interleukin; LPS, lipopolysaccharide; MKK, MAP kinase kinase; MKKK, MAP kinase kinase kinase; pSR, pSuperRetro; TLR, Toll like receptor; Toll/interleukin-1 receptor, TIR domain; wt, wild type.

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INTRODUCTION

The intercellular levels of interleukin-1 (IL-1) increase during inflammatory processes, eliciting a wide array of biological activities [1]. This cytokine acts through the IL-1 receptor (ILR), which belongs to the IL-1R/TLR (Toll-like receptor) superfamily [2]. Members of this receptor superfamily possess a Toll/interleukin-1 receptor domain (TIR domain) in their cytoplasmatic tail, which is required for the recruitment of adapter proteins to transduce signals. In recent years, it has been established that each IL-1R/TLR receptor has a distinct specificity for the different

adapter proteins [2–4]. The common adapter protein, MyD88, is associated to the IL-1R/TLRs through their TIR domains. In turn, MyD88 recruits a signalling complex to the receptor and through its death domain, the serine/threonine kinase IRAK [5–8]. Once IRAK is incorporated into the receptor complex, it becomes highly phosphorylated and activated, making it capable of recruiting TRAF6 to this complex in response to IL-1 [8–11]. Subsequently, and in conjunction with a heterodimeric ubiquitin-conjugating enzyme, TRAF6 catalyzes the addition of unique poly-ubiquitin chains to the kinase TAK and to TRAF6 itself [12,13]. Once activated, TAK forms a complex with several TAB proteins [14–16] and it can activate the IKK complex and several MKKs by phosphorylation. These activations can in turn promote the degradation of IκBα and the activation of the p38 MAPK and JNK pathways [13,17–19]. The human Cot proto-oncogene was originally identified as a C-terminally truncated protein [20]. This disruption of the last coding exon of the human Cot gene or truncation of its murine homologue tpl-2 induces cell transformation [20–23]. Wild type (wt) Cot/tpl-2 is a protein with a short half-live [23,24]. However, the truncation of the C-terminal increases both its specific activity as well as the stability of the protein [21,23], explaining the enhanced transformation capability of the truncated protein. The Cot/tpl-2 gene encodes a MAP kinase kinase kinase (MKKK) that was originally reported to be capable of triggering several MAP kinase cascades in overexpression studies, namely those leading to activation of the MAP kinases: ERK1/ERK2, JNK, p38γ and ERK5 [25–29].

In macrophages, endogenous Cot is the unique MKKK1 that activates ERK1/ERK2 in response to lipopolysaccharide (LPS) [30–33]; however, the activation of JNK and p38γ MAP kinases is unchanged in macrophages from Cot/tpl-2 knockout mice in response to this extracellular stimulus [31]. Cot is also susceptible to be activated in macrophages, in response CpG-DNA stimulation [34] and in B cells, in response to anti-CD40 or TNFα [33]. Recent studies have identified that in resting macrophages Cot protein forms a stable and inactive complex with p105 NFκB, [30,35]. In LPS-stimulated macrophages activated IKKβ phosphorylates p105 NFκB that is then rapidly degraded by the proteasome pathway to p50 NFκB. Dissociated Cot is then capable to activate MKK1 and thus ERK1/ERK2. Subsequently, Cot is also rapidly degraded [24,30,35]. We show here that Cot is the only MKKK involved in the activation of the ERK1/ERK2 pathway in response to IL-1 and that Cot activity is essential for the induction of the levels of IL-8 and MIP-1β mRNA in response to IL-1. We also demonstrate that the IL-1 activation of the Cot-MKK1- ERK1/ERK2 pathway is dependent, as described for the activation of other MAPKs pathways by this cytokine,

of TRAF6. Besides, we also provide evidence that Src kinase activity is specifically involved in the activation of the Cot-MKK1-ERK1/ERK2 pathway.

MATERIALS AND METHODS.

Plasmids and siRNAs — The Cot siRNA containing the sequence 5′ggaccaugguugucaucatt-3′ (GGUCot siRNA) was provided by Ambion and a second Cot siRNA (UCU CotsiRNA), with the sequence 5′-ucuuuaugcaagugaagag.dtdt-3′, as well as the control siRNA were provided by Dharmacon. In order to construct two different pSR Cot siRNA plasmids, two 64-bp primer pairs (368D/368R and 525D/525R) were designed according to the instructions of the oligo Engine company [36]. Each primer pairs contained a 19-nt sequence homologous to the human Cot cDNA sequence. The two 19-bp sequences from the human Cot gene chosen were 5′-tagattccgatgttctcct-3′ and 5′-gccatctgatgtggaaatc-3′, which correspond to nucleotides 368–386 and 525–543 of the human Cot coding sequence, respectively. The annealed and phosphorylated 368D/368R and 525D/525R primers were inserted into a BglII and HindIII digested pSuperRetro (pSR) vector. When transfected into mammalian cells, these two pSR 368 and pSR 525 plasmids express two different Cot siRNAs and confer resistance to puromycin. The pEF-BOS-GST ERK2 construct was generated by digesting the pCDNAIII HA-ERK2 plasmid with BamHI and subcloning the HA-ERK2 insert into the pEF-BOS-GST vector [37], previously digested with BamHI. The plasmid pcINEO HA-Cot wt has been described previously [23]. We thank Prof. Goeddel for the pRK5 Flag-TRAF6 and pRK5 Flag-TRAF6DN (Δ289–522) constructs [10], Piero Crespo for the pCDNAIII HA-ERK2 and pCNAIII, MKK1 S218E, S222E (constitutive active MKK1, MKK1 EE) constructs, andMiguel Angel Iñiguez and Manuel Fresno for the GAL4 Luc and GAL4 Elk constructs.

Cell culture, transfection and stimulation—Hela and HEK cells were maintained in an atmosphere of 95% air, 5% CO2, in DMEM supplemented with 10% heated-inactivated foetal bovine serum (FBS, Hy-Clone), 100 U/ml penicillin and 100 μg/ml gentamicin. Primary human synoviocytes (from six different patients and generously provided by Dr. Eugenia Miranda, Dpto. de Reumatología del Hospital “La Paz”, Madrid) were maintained in RPMI supplemented with

10% FBS, 100 U/ml penicillin and 100 μg/ml gentamicin, and they were used between passages 3 and 7. The human endothelial HMEC-1 cell line was generously provided by the 'Center for Disease Control and Prevention', in Atlanta and was maintained in MCDB131 (Gibco), 15% FBS, 10 mM glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml gentamicin. The day prior stimulation the different cells were plated at a density of 6×105 cells/60 mm plate and maintained in the same incubation media with the exception that the concentration of FBS was reduced to 0.1%.

After 18–24 h in this media, cells were stimulated with 20–40 ng/ml of IL-1 (Roche) or with 500 ng/ml of LPS (Sigma) for the times specified in the figure legends. Where indicated the inhibitors used were added prior to stimulation with IL-1 as follows: 10 μM MKK1 inhibitor U0126 (Promega) 1 h previously; 1 μg/ml herbimycin A (HerbA, Calbiochem) 3 h previously; 100 μM IKK inhibitor SC-514 (Calbiochem) 1 h previously; 20 μM MG132 (Sigma) 1 h previously; 10 μM PP1 analog (PP1, Calbiochem) or 10 μM PP2 (Calbiochem) 30 min previously. Co-transfection in Hela cells, control siRNA to a final concentration of 100nM or UCU Cot siRNA to a final concentration of 25 or 100 nM as indicated by Dharmacon were transfected together with GST-ERK2 (3 μg), using the lipofectamine reagent (Invitrogen). To this purpose, cells were plated at a density of 6×105 cells/60 mm plate. After 72 h of transfection, cells were replated at a density of 6×105 cells/60 mm plate and incubated with DMEM with 0.1% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml gentamicin and maintained for at least 18–24 h prior stimulation. To transfect GST-ERK2 (7 μg), together with wt TRAF6 (7 μg) or TRAF6 DN (7 μg), Hela cells were first plated the day before transfection at a density of 8×105 cells/90mm plate. An aliquot of 2×10^6 cells was replated 48 h after transfection in 90 mm plates with DMEM with 0.1% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml gentamicin, and maintained for at least 18–24 h in this medium prior stimulation. Hela cells were also transfected with pSR (5 μg), pSR 368 (5 μg), pSR 525 (5 μg) or with pSR 368 (2.5 μg) plus pSR 525 (2.5μg) as indicated above. After 24 h of the transfection, the cells were rinsed twice with PBS, and incubated for a further 24 h in DMEM with 10% (v/v) heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml gentamicin plus 1 μg/ml puromycin. Subsequently, the concentration of heat-inactivated FBS was decreased to a concentration of 0.1% (v/v) for 18–24 h prior to stimulation. After stimulation, the medium was removed by aspiration, the cells were rinsed with ice cold PBS, and they were solubilized in ice cold lysis buffer (50 mM Tris-acetate (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium

glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol and acomplete proteinase inhibitor mixture (1 tablet/50 ml, Roche). The samples were then snap frozen in liquid nitrogen and stored in aliquots at −70 °C until they were analysed. Cell extracts were thawed at 0 °C, centrifuged for 10 min at 24,000×g to pellet the debris and the protein concentration was determined by the method of Bradford (Bio-Rad). HEK293 cells in 90 mm plates were transfected with 5 μg of pcINEO HA-Cot wt together with either pSR (5 μg), pSR 525 (5 μg), pSR 368 (5 μg) or pSR 525 (2.5 μg)/pSR 368 (2.5 μg) together as described previously [23]. After 24 h, the cells were incubated for a further period of 24–36 h in the same medium plus 1 μg/ml puromycin, and then cells were collected.

Cot assay and Western blot analysis—The Cot assay was performed as indicated previously [32] except that Cot was immunoprecipitated from 0.5 mg of the different Hela cell extracts using the anti-tpl-2/Cot antibody (Santa Cruz). Unless otherwise indicated, Western blots were performed using 35 μg of protein from cell extracts that were resolved on 10% SDS/polyacrylamide gels, transferred to PVDF membranes and probed with anti-Cot (Calbiochem or Santa Cruz), anti-IκBαand anti-FLAG(Santa Cruz) antibodies, or with the following antibodies from Cell signaling: anti-P-ERK1/ERK2, anti-ERK2, anti-P-p38 MAPK and anti-PJNK.Antibody binding was revealed using a chemiluminescent method (ECL, Amersham-Pharmacia-Biotech).

Transactivation assays— Hela cells (5×105) were transfected in 60 mm plates with 1.3 μg Gal4 Luc plus 2.17 μg Gal4 Elk together with 2.17 μg of wt TRAF6, TRAF6 DN, MKK1 EE or with the empty vector, and with or without the different siRNAs (100 nM). The cell medium was replaced 18 h after transfection with DMEM with 0.1% (v/v) heat-inactivated FBS and, 24 h later, the cells were stimulated for 5 h with 40 ng/ml IL-1 following a prior pre-incubation with different inhibitors when necessary. The luciferase activity was then measured as indicated previously [38].

Quantitative reverse transcription-PCR assay—Total RNA from Hela cells transfected with pSR, or with both pSR 525 and pSR 368 plasmids as indicated above, were stimulated with IL-1 for different periods of time. The RNA from these cells was then isolated and reverse transcribed as described previously [39]. Quantitative PCR was performed and analysed using MIP-1β, IL-8 and human 18S specific probes for real time PCR (Applied Biosystems) according to manufacturer's instructions.

RESULTS.

Cot is the only MKKK that up-regulates ERK1/ERK2 in response to IL-1— Stimulation of Hela cells for 15 min with 40 ng/ml IL-1 but not with PMA(0.4 μg/ml) increased Cot activity by about 10 fold (Fig. 1A). To investigate whether Cot is the only MKKK that up-regulates ERK1/ERK2 in response to IL-1, we studied how co-transfection of two different Cot siRNAs might affect Elk mediated transactivation induced by IL-1. First, we analysed to which extent the ERK1/ERK2 pathway is involved in the IL-1-induced Elk mediated transactivation, to this end cells transfected with the GAL4 Elk and GAL Luc plasmids were pre-incubated for 30 min with U0126 (10 μM) prior the5 h stimulation with IL-1. The pre-treatment of cells with U0126 inhibited IL-1-induced Elk transactivation by approximately 90% (data not shown), indicating that the Elk transactivation by IL-1 is almost exclusively regulated by the ERK1/ERK2 pathway. Indeed, the co-transfection of Hela cells with UCU Cot siRNA (100 nM) or GGU Cot siRNA (100 nM) as well as with the GAL4 Elk and GAL Luc plasmids, blocked the Elk mediated transactivation on stimulation with IL-1 (Fig. 1B). Two isoforms of Cot protein corresponding to two alternative translation initiation sites of the murine Cot/tpl-2 gene (M1 and M30) has been described in RAW macrophagic cell line[30], although only Cot (M1) seems to plays a role in the activation of ERK1/ERK2 pathway in response to LPS[24,30,40]. To further establish that Cot regulates ERK1/2phosphorylation induced by IL-1 and considering that in our conditions assay only a 70% of the cells are positively transfected, we decided to co-transfect UCU Cot siRNA (25 or 100 nM), as well as control siRNA (100 nM), together with GST-ERK2 and to determine endogenous Cot protein levels as well as phosphorylation of GST-ERK2 in response to IL-1 stimulation.Western blot analysis of Hela cells co-transfected with the control siRNA and utilizing an antibody against the C-terminal of Cot revealed the expression of a major band of molecular mass of about 58 kDa that correspond to Cot (M1) (Fig. 1C). A minor band with a lower molecular mass was also observed. This band could correspond to a degradation product of Cot (M1) or to the second isoform of Cot protein with the translation initiation site at M30. Cells transfected with UCU Cot siRNA exhibited a significant lower Cot protein (M1) expression (Fig. 2B). Interestingly, the expression levels of the minor lower molecular mass band did not decrease to the same extent than Cot (M1) protein levels. The co-transfection of UCU Cot siRNA (25 or 100 nM), but not co-transfection of control siRNA (100 nM), blocked the phosphorylation of the

co-transfected GST-ERK2 in response to IL-1 stimulation (Fig. 1C). These data suggest that Cot is the only MKKK that regulatesERK1/ERK2 activation in response to IL-1.

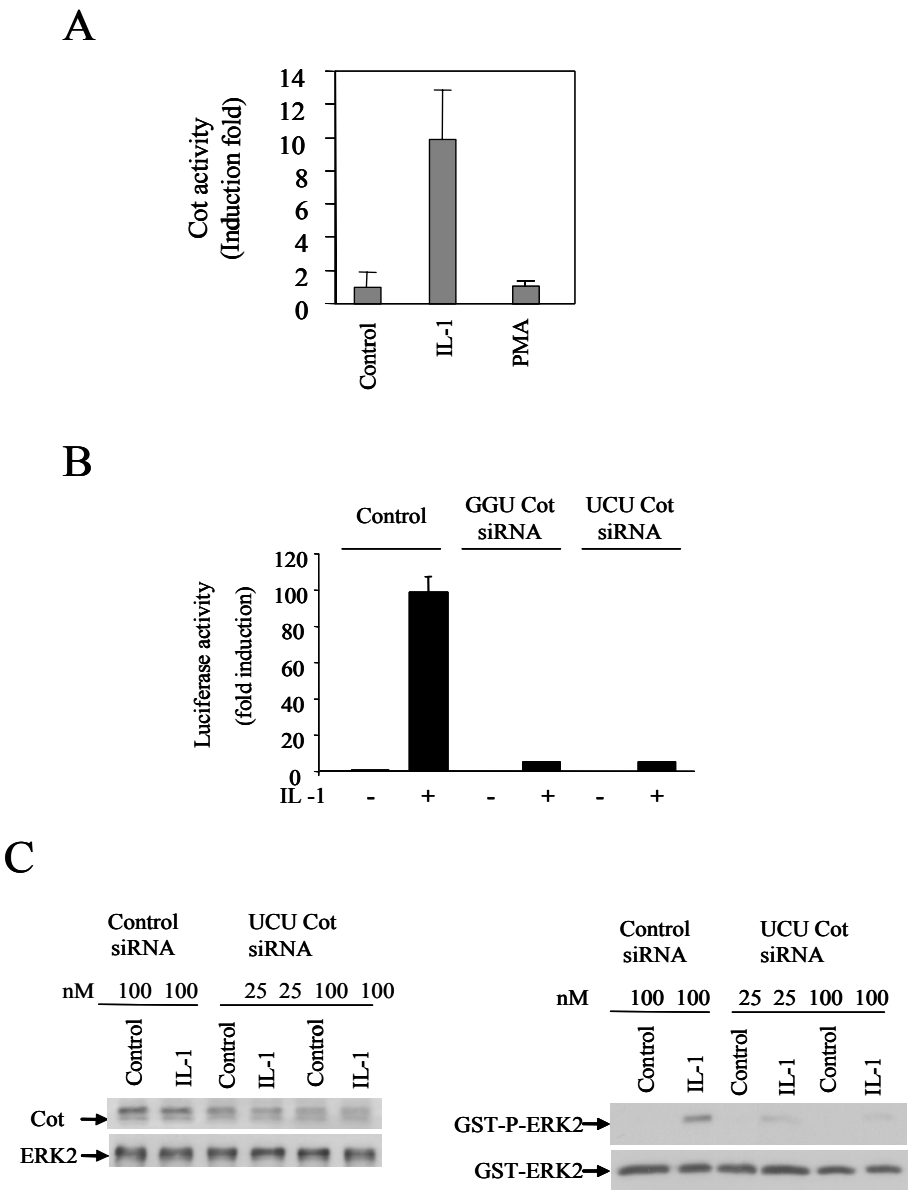

Fig. 1. Cot is the sole MAPKKK that regulates the ERK1/ERK2 pathway in response to IL-1. (A) Hela cells were stimulated for 15 min with 40 ng/ml of IL-1 or with 0.4 µg/ml of PMA. Cot was immunoprecipitated from the lysed cells and assayed for Cot kinase activity as described under Materials and methods. The results are represented as the mean±S.E.M. of three measurements from three separate experiments. (B) Hela cells were transfected with Gal4 Luc (2.17 µg) and Gal4 Elk (2.17µg) together with control siRNA (100 nM), GGU Cot siRNA (100 nM) or with UCU Cot siRNA (100 nM), as described under Materials and methods. After 48 h of transfection, cells were stimulated for 5 h with 40 ng/ml of IL-1 and the luciferase activity was measured. The data represent the % increase of luciferase activity in triplicate determinations ±S.D., the values of control siRNA transfected cells being attributed the value of 1 and when stimulated with IL-1 a value of 100. (C) Helacells plated in 60 mm plate were co-transfected with GST-ERK2 (3 µg) together with control siRNA (100 nM) or with UCU Cot siRNA (25 or 100 nM). After 72 h, the cells were stimulated with 40 ng/ml IL-1 for 20 min and the cell extracts were then analysed by Western blotting utilizing anti-Cot (Santa Cruz), anti-P-ERK1/ERK2 and anti-ERK2. Similar results were obtained in three independent experiments.

The IL-1 induction of IL-8 and MIP-1β mRNA levels are dependent of Cot expression—To analyze the role of Cot activity in the IL-1 signalling, we decided to repress Cot expression by the transfection of two different Cot pSR siRNA plasmids, one expressing a siRNA corresponding to the nucleotide coding sequence 368–386 of human Cot (pSR 368) and the second expressing another siRNA corresponding to the nucleotide sequence 525–543(pSR 525). The transfection of these two plasmids confers to the positive transfected cells resistance to puromycin and thereby only the positive transfected cells can be selected by treatment with this antibiotic. The capacity of these pSR constructs, pSR 525 and pSR 368, to block Cot expression was first tested in HEK293 cells co-transfected with pcINEO HA-Cot wt together with empty pSR, pSR 368, pSR 525, or with both pSR 368 and pSR 525 plasmids. The levels of Cot mRNA in quantitative RT-PCR assays were 75–85% lower in cells transfected with pSR 368 or pSR 525 than in cells transfected with the empty pSR, but the co-transfection of both plasmids pSR 368 and pSR 525 together reduced Cot mRNA expression levels to about a 100% when compared with control cells (data not shown). In agreement with these data, less Cot protein was found in cells co-transfected with pcINEO HA-Cot wt together with pSR 368 or with pSR 525 than in cells cotransfected with pcINEO HA-Cot wt together with empty pSR (Fig. 2A), but the co-transfection of both pSR 525 and pSR 368 plasmids together with pcINEO HA-Cot wt almost completely blocked the accumulation of Cot protein (Fig. 2A). Moreover, while overexpression of Cot in HEK293 cells triggered phosphorylation of endogenous ERK1/ERK2 (Fig. 2A,[23]), this activation did not occur when cells were co-transfected with pcINEO HA-Cot wt together with pSR 368 and pSR 525 (Fig. 2A). These data indicate that the transfection of both pSR 368 and pSR 525 together produces a more effective inhibition of Cot protein expression than transfection of either of the two plasmids alone. Therefore, we next tested if the co-transfection of both plasmids together in Hela cells regulates endogenous Cot expression. The co-transfection of both plasmids pSR 368 and pSR 525 together in Hela cells dramatically reduced Cot mRNA expression levels (data not shown). Thereby, we decided also to determine how the co-transfection of both plasmids pSR 368 and pSR 525 affects the expression of endogenous Cot protein in Hela cells. The transfection of both pSR 368 and pSR 525 plasmids in Hela cells decreased to almost undetectable levels Cot (M1) expression (Fig. 2B). As observed previously in Hela cells transfected with the UCU Cot siRNA, the expression levels of the minor lower molecular mass band did not decrease to the same extent than Cot (M1) protein levels (Fig. 2B). On the other hand, Hela cells transfected with both pSR 368 and pSR 525 plasmids together and resistant to the puromycin treatment did not longer show any activation of endogenous ERK1/ERK2 in response to IL-1stimulation (Fig. 2C).

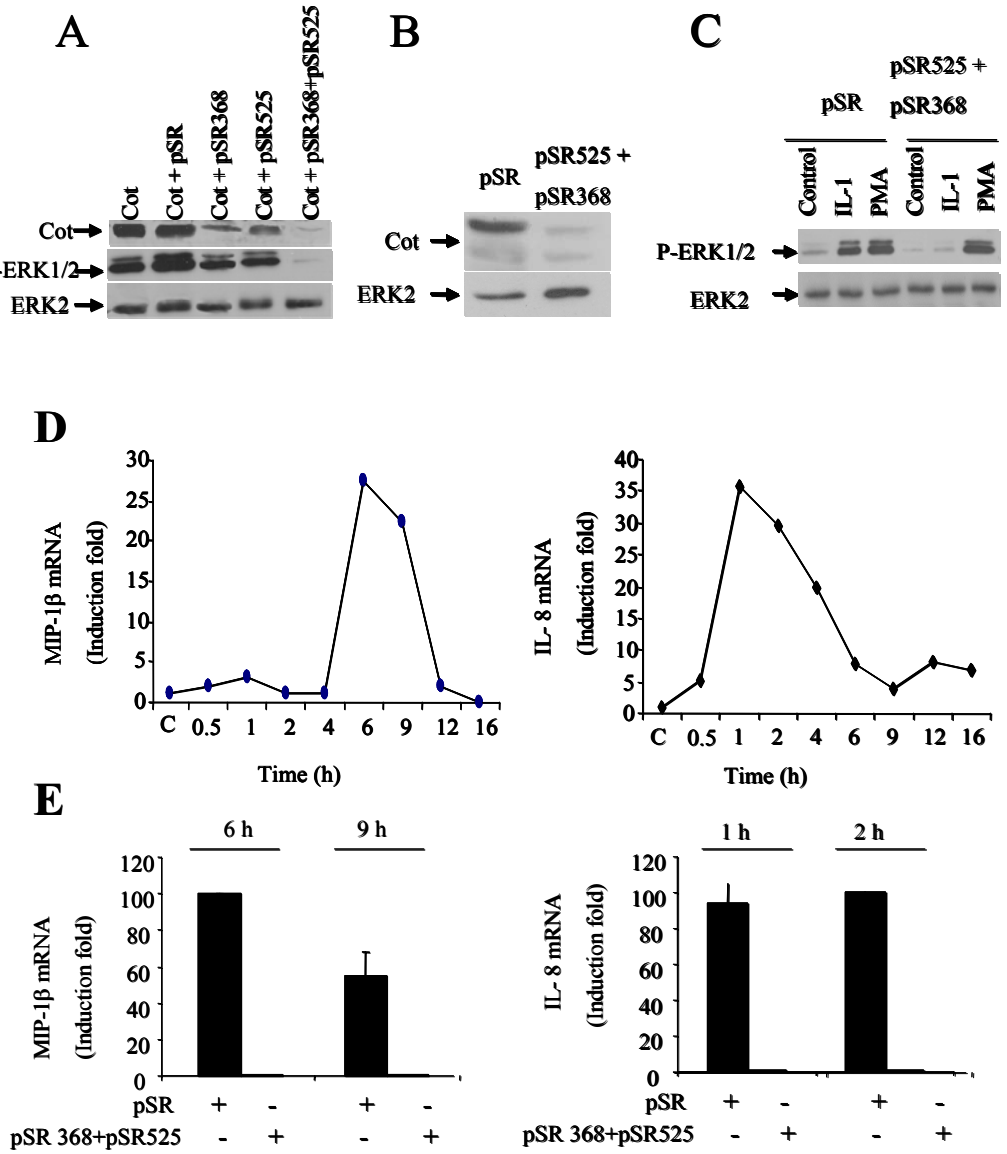

Fig. 2. Cot expression is essential to increase MIP-1β and IL-8 mRNA levels by IL-1. (A) HEK293 cells were transfected with 5 μg of pcINEO HA-Cot wt together with the empty pSR vector (5 μg), pSR 368 (5 μg), pSR 525 (5 μg) or with pSR 368 (2.5 μg) and pSR 525 (2.5 μg) together. Cell lysates were analysed by Western blot and probed with anti-P-ERK1/ERK2 and with anti-Cot (Calbiochem). As a loading control, the total amounts of ERK-2 were determined. The figure shows one of the three experiments performed. (B) Hela cells were transfected with a mixture of pSR 525 (2.5 μg) together with pSR 368 (2.5 μg) or with empty vector (5 μg); 24 h after transfection, cells were treated with puromycin (1 μg/ml) and 48 h after transfection collected. Cot protein expression levels were determined by Western blot analysis utilizing anti-tpl-2/Cot (Santa Cruz) as described under Materials and methods. As a loading control, the total amounts of ERK-2 were determined. Similar results were obtained at least three times. (C) Puromycin resistant Hela transfected cells, obtained as described in (B), were stimulated for 20 min with 40 ng/ml of IL-1 or with 0.4 μg/ml of PMA. The lysates were analysed by Western blotting and probed with anti-P-ERK1/ERK2 and anti-ERK2. Similar results were obtained in three different experiments. (D) Hela cells were stimulated with IL-1 (40 ng/ml) for the times indicated in the figure. The IL-8 and MIP-1β mRNA levels were determined by RT-PCR analysis as indicated under Materials and methods. The figure shows a representative experiment of the three performed for each chemokine. (E) Hela cells were transfected with pSR (5 μg) or with pSR 368 (2.5 μg) and pSR 525 (2.5 μg), and the cells resistant to puromycin were stimulated with IL-1 for 1 h and 2 h to determine IL-8 mRNA levels or for 6 h and 9 h to determine the MIP-1β mRNA levels. The graph shows the means of three experiments performed in duplicate.

As expected, phosphorylation and activation of ERK1/ERK2 in response to PMAwas not affected by co-transfection of the pSR 525 and pSR 368 plasmids (Fig.2C), this phenomenon being mediated by the MKKK c-RAF [32].

Taking advantage of the possibility to select only the Hela cells that do not express Cot protein, we decided to examine the role of Cot in the IL-1 signalling. The induction of genes involved in inflammatory processes is one of the key functions of IL-1. To assess the role of Cot in IL-1 signalling, we determined the levels of IL-8 and MIP-1β mRNA by quantitative RT-PCR analysis, these being two chemotactic stimuli for neutrophils and monocytes, respectively. Hela cells were stimulated with IL-1 (40 ng/ml) for different times and the levels of IL-8 and MIP-1β mRNA expression were determined by quantitative RT-PCR analysis. Maximum expression of IL-8 mRNA levels was observed 1–2 h after stimulation with IL-1, while stimulation with IL-1 over 6–9 h was required to induce maximal levels of MIP-1β mRNA (Fig. 2D). This IL-1 induction of both mRNA transcripts was repressed by exposure to U0126 (data not shown). Therefore, we tested whether the inhibition of Cot expression also blocked the induction of IL-8 and MIP-1β mRNA expression. To this end, cells were transfected with both pSR 368 and pSR 525 together or with pSR, and the puromycin resistant cells were stimulated with IL-1 for different times. In transfected cells stimulated for 0 h, 6 h and 9 h, the MIP-1β mRNA levels was determined, while the mRNA levels of IL-8 was determined in cells stimulated for 0 h, 1 h and 2 h. The inhibition of Cot expression by pSR 368 and pSR 525 completely abolished the induction of IL-8 and MIP-1β mRNA expression following IL-1 stimulation for the times tested (Fig. 2E). All these data indicate that Cot plays an essential role in the inflammatory processes triggered by IL-1, due to its capacity to regulate the ERK1/ERK2 dependent expression of genes involved in the inflammatory signal.

TRAF6, IKKβ and p105 NFκB mediate the ERK1/ERK2 activation and Cot degradation in response to IL-1— In resting macrophages, Cot is bound to p105 NFκB forming a complex that stabilizes Cot protein [30]. In LPS-activated macrophages, IKKβ a component of the IKK complex phosphorylates p105 NFκB, this phosphorylation triggers its partial degradation to p50 NFκB in a proteasome dependent manner,dissociated Cot is also subsequently degraded [24,35]. We firstdecided to study if IL-1 also triggers Cot degradation in Hela cells. To this end, cell were stimulated for different times with 40 ng/ml IL-1 and ERK1/ERK2 activation as well as Cot protein expression was analyzed. Maximal phosphorylation of ERK1/ERK2 was observed 15–30 min after IL-1 stimulation and a decrease in the Cot (M1) protein expression levels was observed

30–45 min after the stimulation, when the MKK1-ERK1/EKR2 pathway is already switched on (Fig. 3A).

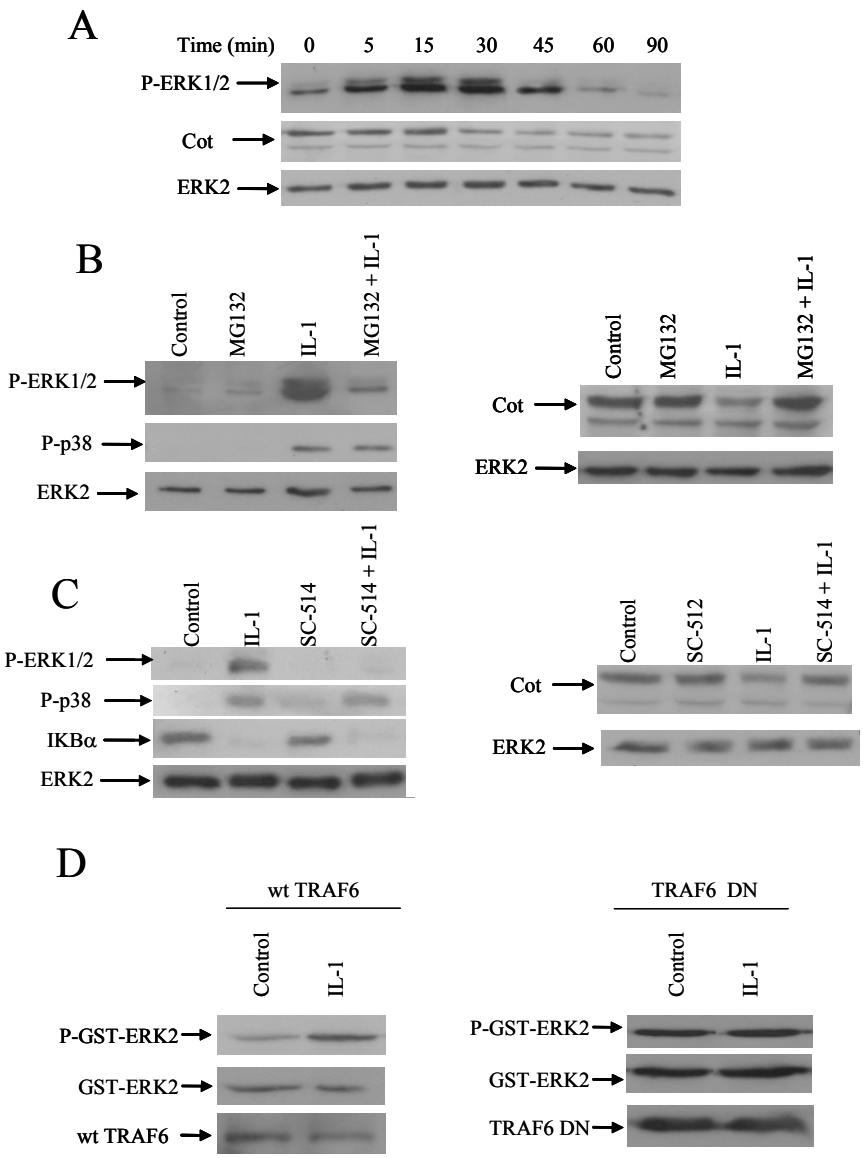

Fig. 3. IKKβ and TRAF6 are required for ERK1/ERK2 activation by IL-1. (A) Hela cells were stimulated for 0, 5 min, 15 min, 30 min, 45 min, 60 min and 90 min with 40 ng/ml IL-1. Phospho-ERK1/ERK2, Cot and ERK2 expression levels were determined by Western blot analysis. Cot expression levels were determined utilizing anti-tpl-2/Cot from Santa Cruz. (B) Hela cells were pre-incubated or not with the proteasome inhibitor MG132 (20 μM) for 1 h and stimulated for 20 min (left panel) or for 1 h (right panel) with 40 ng/ml IL-1. ERK1/ERK2 and p38 MAPK activation was determined byWestern blot analysis utilizing anti-P-ERK1/ERK2 and anti-P-p38 MAPK, after 20 min IL-1 stimulation. Cot protein expression levels were analyzed by Western blot analysis utilizing anti-tpl-2/Cot (Santa Cruz), after 1 h IL-1 stimulation in the presence or absence of MG132 (20 μM). As a loading control, the total amounts of ERK-2 are also shown. (C) Hela cells were stimulated with 40 ng/ml IL-1 for 20 min with or without a prior 1 h exposure to the IKKβ inhibitor SC-514 (100 μM). Cell lysates were analysed by Western blotting and probed with anti-P-ERK1/ERK2, anti-P-p38 MAPK, anti-IκBα and anti-ERK-2. Cot protein expression levels were analyzed by Western blot analysis utilizing antitpl-2/Cot (Santa Cruz), after 1 h IL-1 stimulation in the presence or absence of MG132 (20 μM). (D) Hela cells were co-transfected with GST-ERK2 together with wt TRAF6 or TRAF6 DN. After 72 h, cells were stimulated with 0 ng/ml or 40 ng/ml of IL-1 for 20 min, and the cell extracts were then analysed by Western blotting using anti-PERK1/ERK2, anti-ERK2 and anti-Flag antibodies. (A, B, C and D) Similar results were obtained in three independent experiments.

The pre-incubation of Hela cells with MG132 (20 μM) prior a 20 min stimulation with IL-1 blocked ERK1/ERK2 activation (Fig. 3B). MG132 pre-incubation also prevented ERK1/ERK2 activation in response to IL-1 in human primary synoviocytes and in the human endothelial cell line HMEC-1 (data not shown). These data could indicate that pre-incubation with MG132 blocks the proteolysis of p105-NFκB and thereby Cot dissociation, as described previously in macrophages. In fact, the pre-incubation with MG132 also blocked the subsequent Cot protein degradation detected after 60 min stimulation with IL-1 (Fig. 3B). These data also agree with the fact that the pre-incubation with SC-514 (100 μM), an inhibitor of IKKβ [41–44], also prevented the activation of ERK1/ERK2 by IL1 (Fig. 3C).

The pre-incubation with this inhibitor did not block the degradation of IκBα induced by IL-1 (Fig. 3C), providing evidence of the specificity of SC-514 in blocking IKKβ but not IKKα activity. As expected, the pre-incubation of Hela cells with SC-514 also prevented the subsequent Cot degradation after IL-1 stimulation (Fig. 3C). The oligomerization of TRAF6 in response to IL-1 stimulation leads to the activation of the IKK complex as well as to the activation of the JNK and p38 MAP kinases pathways [13,45]. To study the role of TRAF6 in the activation of the ERK1/ERK2 pathway in Hela cells after IL-1 stimulation, cells were co-transfected either with a dominant negative construct of FLAG-TRAF6 (TRAF6 DN) or with a wt FLAG-TRAF6 construct together with GST-ERK2. An increase in the phosphorylation state of GST-ERK2 was observed, when the cells co-transfected with wt TRAF6 were stimulated with IL-1. In contrast, no increase in GST-ERK2 phosphorylation was observed in cells expressing the TRAF6 DN after stimulation with IL-1 (Fig. 3D). All these data indicate that the activation of the ERK1/ERK2 pathway, as described previously for the IL-1 activation of other MAP kinases pathways, is mediated by TRAF6. On the other hand, the activation of the Cot-MKK1-ERK1/ERK2 but not the activation of p38 MAPK in response to IL-1, is dependent of IKKβ and proteasome activities (Fig.3B and C).

Involvement of Src kinase activity in the activation of the Cot-MKK1-ERK1/ERK2 pathway—

Pre-incubation of Hela cells for 3 h with 1 μg/ml HerbA, a general and irreversible inhibitor of protein tyrosine kinases, blocked the activation of ERK1/ERK2 induced by IL-1 in Hela cells. Moreover, the pre-incubation for 30 min with two specific inhibitors of Src protein tyrosine kinases such as PP2 (10 μM) or PP1 (10 μM) also impaired the activation of ERK1/ERK2 by IL-1 (Fig. 4A).

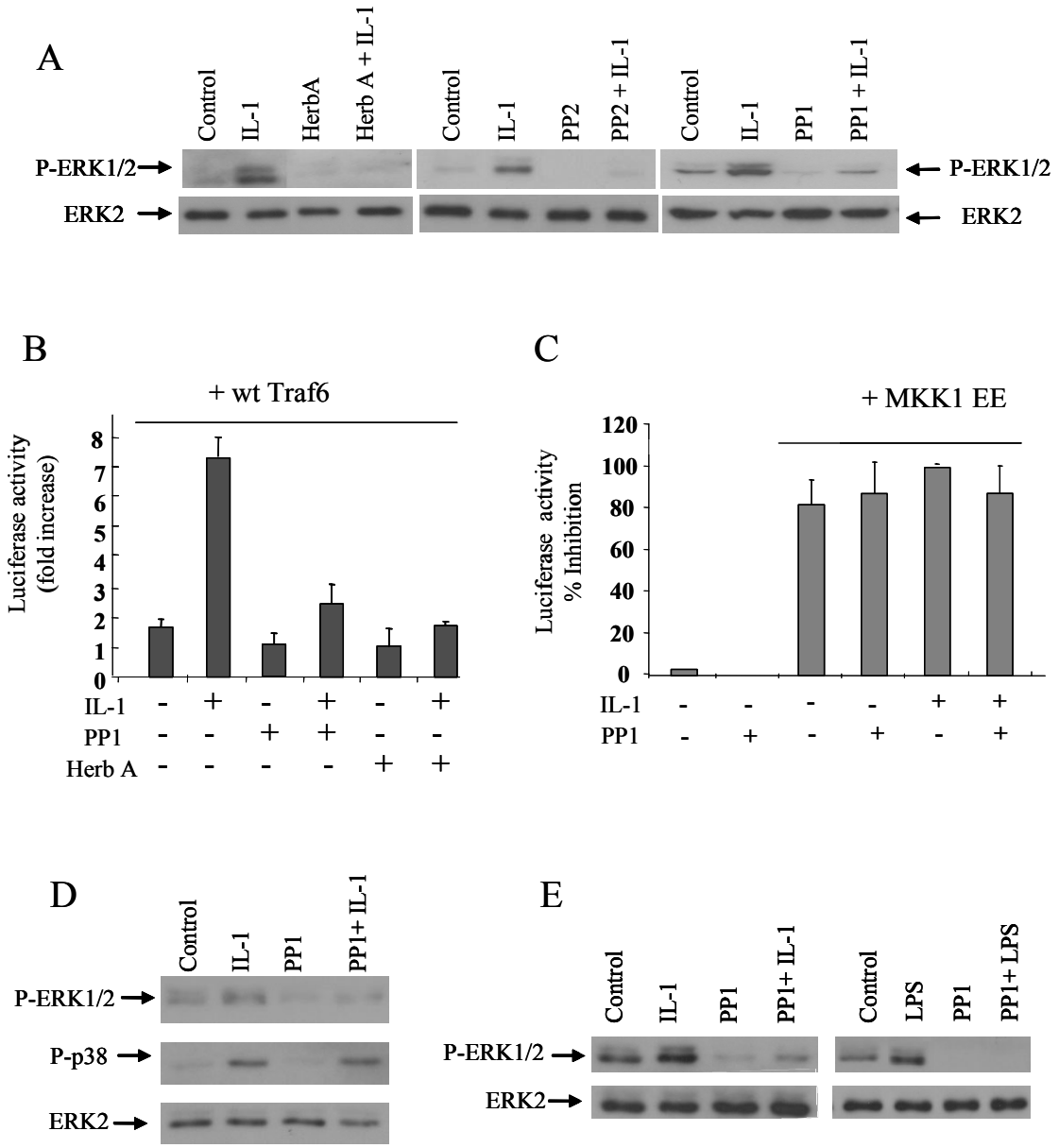

Fig. 4. Inhibitors of Src kinase activity inhibit ERK1/ERK2 activation induced by IL-1 or by LPS. (A) Hela cells were pre-incubated with HerbA (1 μg/ml), PP2 (10μM) or PP1 (10 μM), and then stimulated with IL-1 (20 ng/ml) for 20 min. The cell extracts were analysed by Western blotting with anti-P-ERK1/ERK2 and anti- ERK2. (B) Hela cells were transfected with Gal4 Luc (2.17 μg) and Gal4 Elk (2.17 μg) together with wt TRAF6 (5 μg). After 48 h of transfection, the cells were preincubated with HerbA (1 μg/ml, for 3 h) or with PP1 (10 μM, for 30 min), and then stimulated with 40 ng/ml of IL-1 for 5 h before measuring their luciferase activity. The data show the fold increase of luciferase activity for three determinations, ±S.D. and the value of 1 was attributed to the cells pre-incubated with HerbA and not stimulated with IL-1. (C) Hela cells were transfected with Gal4 Luc (2.17 μg) and Gal4 Elk (2.17 μg) together with MKK1 EE (5 μg) or with empty vector. After 36–48 h of transfection, cells were pre-incubated with PP1 (10 μM) and then stimulated with 20 ng/ml of IL-1 for 5 h before measuring their luciferase activity. The datashow the increase of luciferase activity±S.D. for three measurements and a value of 100 was attributed to the cells stimulated with IL-1 and the value of 1 to cells transfected with empty vector and not stimulated. (D) Synoviocytes were pre-incubated with PP1 (10 μM) and stimulated with IL-1 (20 ng/ml, 20 min) as indicated above. The cell lysates obtained following each treatment were separated by electrophoresis and immunoblotted with anti-P-ERK1/ERK2, anti-P-p38 MAPK and anti-ERK2. (E) HMEC-1 cells were pre-incubated with PP1 (10 μM) and stimulated with IL-1 (20 ng/ml) or with LPS (500 ng/ml) for 20 min. The cell extracts were analysed by Western blotting using anti-P-ERK1/ERK2 and anti-ERK2. In A, D and E, the experiments were performed at least three times with similar results.

The addition of HerbA, as well as PP1, to the cell incubation media also blocked the IL-1-induced Elk mediated transactivation activity (data not shown). Moreover, the inhibition by HerbA, as well as PP1, on the IL-1-induced Elk mediated transactivation activity was also observed in cells cotransfected with wt TRAF6 (Fig. 4B). However, PP1 did not inhibit the Elk mediated transactivation in cells co-transfected with constitutive active MKK1 (MKK1 EE) together with the Gal4 Luc and Gal4 Elk constructs, and stimulated or not with IL-1 (Fig. 4C). Similar data were observed when cells were preincubated with HerbA (data not shown). These data demonstrate that PP1 inhibits Elk mediated transactivation by impairing IL-1 activation of the Cot-MKK1 signalling pathway, discarding the possibility that the pre-incubation with the Src kinase activity inhibitors would enhance the dephosphorylation rate of ERK1/ERK2. The requirement of a Src tyrosine kinase to activate ERK2/ERK2 in response to IL-1 is not specific of Hela cells since the pre-incubation with PP1 also inhibited the IL-1-induced activation of ERK1/ERK2 in primary human synoviocytes, as well as in the HMEC-1 cell line (Fig. 4D). To determine if the requirement of a Src kinase activity to activate the Cot-MKK1-ERK1/ERK2 pathway is specific or not for the IL-1 signalling, and considering that Cot activation has been mainly studied in macrophages in response to LPS, HMEC-1 cells were also pre-incubated with PP1 and stimulated with LPS (500 ng/ml) for 20 min to analyze the ERK1/ERK2 phosphorylation levels. PP1 also blocked the activation of ERK1/ERK2 induced by LPS in HMEC-1 cells (Fig. 4D) and in RAW macrophages (data not shown).

Inhibition of Src kinase activity does not prevent IL-1-induced Cot degradation—The activation of the different MAPKs like p38, JNK and ERK1/ERK2 by IL-1 share some common elements like TRAF6 (Fig. 3C and [13,45]), thereby we decided to study whether PP1 also blocked the IL-1 activation of other MAPKs besides ERK1/ERK2. The PP1 pre-incubation had no effect on the phosphorylation of p38 MAPK and JNK induced by IL-1. Furthermore, PP1 was also unable to block the degradation of IκBα in response to IL-1 (Fig. 5A), indicating that only the ERK1/ERK2 activation is regulated by Src kinase activity in response to this cytokine. Cot activation by LPS is triggered by its dissociation from the Cot-p105 NFκB complex subsequently, Cot undergoes degradation. Thereby, we decided to investigate if the pre-incubation with PP1 also prevented Cot dissociation and thereby its degradation. To this end, Hela cells, as well as human primary synoviocytes, were pre-incubated with or without PP1 during 30 min and after 1 h stimulation with IL-1 the endogenous Cot protein levels were analyzed. Inhibition of the Src kinase activity

did not prevent the decrease in the steady state expression levels of Cot induced by IL-1 (Fig. 5B). These data indicate that the activation of the Cot-MKK1-ERK1/ERK2 pathway requires not only the dissociation of Cot from the Cot-p105 NFκB complex but also a Src kinase activity and that this Src kinase activity does not block Cot dissociation.

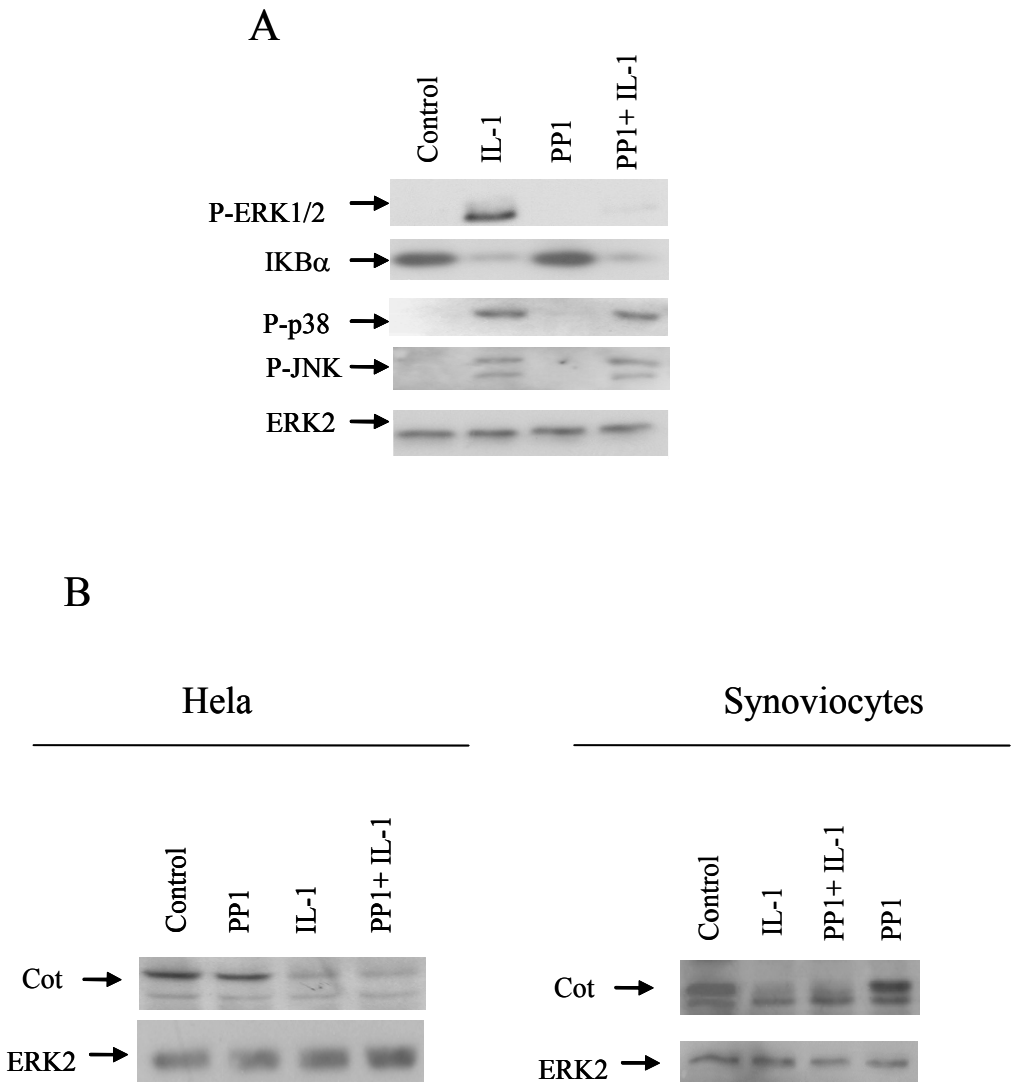

Fig. 5. Inhibition of Src activity does not prevent IL-1-induced Cot degradation. (A) Hela cells were pre-incubated for 30 min with PP1 (10 μM) and then stimulated with IL-1 (20 ng/ml) for 20 min. The cell extracts were analysed by Western blotting with anti-P-ERK1/ERK2, anti-P-p38 MAPK, anti-P-JNK and anti-IκBα. As a control of loading, the total ERK-2 levels were determined. The figure shows one of the three experiments performed with similar results.(B) Hela cells, as well as human primary synoviocytes, were pre-incubated with PP1 (10 μM) and stimulated with IL-1 (20 ng/ml) for 60 min. The cell extracts were analysed by Western blotting using anti-tpl-2/Cot (Santa Cruz) and anti-ERK2. The experiments were performed at least three times with similar results.

DISCUSSION

It has been established that the MKKK Cot plays an important role in intracellular signalling in haematopoietic cells [24,31,34,35]. Our data indicate that Cot activity is also critical in the intracellular signalling in non-haematopoietic cells, thereby Cot should have a role in a diversity of cellular functions. Moreover, the data obtained here also indicate that IL-1 exercises the activation of the ERK1/ERK2 trough the activation of Cot, independently of the cell type. The cytoplasmic tail of the IL-1 receptor has a Toll/interleukin-1 (IL-1) receptor (TIR) domain that structurally, is very closely related to the intracellular region of the TLRs [2]. Indeed, stimulation of the TLR4 by LPS induces a ten-fold increase in Cot activity [32] and in macrophages isolated from Cot/tpl-2 knockout mice, LPS stimulation fails to activate ERK1/ERK2 [31]. ERK activation was impaired in Cot (−/−) B cells and macrophages stimulated with agonistic CD40 antibody or TNF-α [33]. However, stimulation of macrophages through the TLR-9 by CpGDNA increases Cot activity and still induces ERK1/ERK2 activity in macrophages from Cot/tpl-2 knock-out mice [34].This activation of ERK1/ERK2 indicates that Cot is not the only MKKK responsible for activating the classical MAP kinase pathway in response to CpG-DNA. Therefore, in TLR intracellular signalling, the importance of Cot activation tofurther stimulate ERK1/ERK2 depends on the nature of the TLR activated. In this paper, we demonstrate that the increase in Cot/tpl-2 activity promoted by IL-1 is essential for the activation of ERK1/ERK2 and that Cot activity controls the functions of IL-1 that are mediated by ERK1/ERK2. Indeed, we have shown here that Cot regulates the expression of IL-8 and MIP-1β,two chemokines that play an important role in the development of inflammation. Meanwhile this manuscript was in preparation, it has been published that TNF-α requires the transducer protein TRAF2 [46] and not TRAF6 to induce Cot dependent ERK1/ERK activation, as shown here. Moreover, TNF-α requires Cot activity not only for the activation of ERK1/ERK2 but also for the activation of other MAPK such us p38 and JNK [46]. However, the fact that none of the different inhibitors MG132, SC-514 or PP1, that inhibited different steps involved in the activation of Cot-MKK1-ERK1/ERK2 by IL-1, did not block the activation of p38 or JNK indicate that Cot plays a different role in the activation of the different MAPKs in response to IL-1 or in response to TNF-α. In this context, it should be mention that in the LPS signalling Cot only activates the ERK1/ERK2 pathway since the activation of JNK and p38γ MAP kinases is unchanged in macrophages from Cot/tpl-2 knockout mice in response to this extracellular stimulus [31].

According to its sequence, Cot could have an alternative translational initiation site on a second methionine (M30), resulting in the expression of two Cot isoforms. Western blot analysis of Hela cells utilizing an antibody against the C-terminal of Cot revealed the expression of a major band of molecular mass of about 58 kDa that correspond to Cot (M1) and a minor band that could correspond to Cot (M30). Similar results have been obtained in the RAW macrophagic cell line utilizing this antibody against the C-terminal of Cot [30]. However, it has been proposed that only Cot (M1) plays a role in the activation of ERK1/ERK2 pathway in response to LPS [24,30]. In fact, we have previously shown that Cot immunoprecipitated from LPS-stimulated RAW cells extracts exhibited 10 times more Cot activity than Cot immunoprecipitated from control RAW cell extracts, and only one radiolabelled band, corresponding to Cot (M1), could be detected in these immunoprecipitation experiments [32].

Regarding the intracellular mechanism involving Cot-MKK1-ERK1/ERK2 activation by IL-1, our data indicate, as described previously in macrophages, that in resting cells Cot levels are stabilized by the association with p105-NFκB and its dissociation is required to induce the activation of ERK1/ ERK2. In fact, Western blot analysis with anti p50-NFκB antibody of immunoprecipitated Cot in low salt conditions from resting Hela cells, but not in IL-1-stimulated Hela cells, revealed a band of 105 kDa that could correspond to p105-NFκB (data not shown). One of our most important findings is that the activation of the Cot-MKK1-ERK1/ERK2 pathway is also mediated by a Src tyrosine kinase. Other tyrosine kinase inhibitors such as AG126, AG490 (tyrphostin B42), AG957 or AG1296 were not able to block the ERK1/ERK2 activation induced by LPS in RAW cells or by IL-1 in Hela cells (data not shown) indicating that tyrosine kinases of the Src family play a specific role in the Cot-MKK1-ERK1/ERK2 pathway activated by IL-1/LPS. In this context, it should be notice that different Src kinases can be activated in response to IL-1 or LPS stimulation [47–53]. We were unable to obtain a clear suppression in the IL-1-induced phosphorylation of co-transfected GST-ERK2 by inhibiting one by one the expression of the different Src tyrosine kinases [54], utilizing siRNAs technology (data not shown). Moreover, co-transfection of Hela cells with a c-Src DN construct together with GST-ERK2 did not inhibit GST-ERK2 phosphorylation induced by IL-1 stimulation (data not shown). These data raise the possibility that different Src tyrosine kinases may be involved in Cot-MKK1-ERK1/ERK2 activation by IL-1. Nevertheless, our data also demonstrate that the Src tyrosine kinase activity does not regulate the dissociation and subsequently degradation of Cot, indicating that not only

Cot dissociation, but other events are also involved in Cot activation, and further studies have to be carried on to identify them. In conclusion, we have shown that Cot activity, induced by IL-1 is the only MKKK activity responsible for the phosphorylation of ERK1/ERK2 and that Cot activity plays an important role in regulating IL-1 functions, such as the up-regulation of IL-8 and MIP-1β mRNA levels. We have also demonstrated that the activation of the Cot-MKK1-ERK1/ERK2 by IL-1 occurs in a specific way, involving the activation of TRAF6 as well as Src tyrosine kinase activity.

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PUBLICACIÓN 3

Cot/tpl-2 es una de las MKKKs que activan la vía de ERK1/ERK2 en respuesta a señales extracelulares implicadas en inmunidad innata, así Cot/tpl-2 se presenta como la única MKKK que activa está ruta en respuesta a IL-1 y LPS, relacionándola directamente con mecanismos de inflamación.

Tanto el gen humano de Cot/tpl-2 como el de su homólogo murino tpl-2 fueron identificados como oncogenes que presentaban una reorganización del extremo 3´ y originaban una proteína truncada/modificada. Así, aunque fisiológicamente Cot/tpl-2 esté asociada a la inmunidad innata, cada vez son más las evidencias que la relacionan con transformación celular y tumorogénesis. Por ejemplo la inserción del virus de tumores de mama de ratones (MMTV) en el último intrón del gen de Cot/tpl-2 se ha relacionado con la transformación celular en células de glándula mamaria y también se ha establecido una relación directa entre altos niveles de ARNm de Cot/tpl-2 y el desorden granular linfocítico.

En este trabajo hemos demostrado que una sobreexpresión de la forma truncada/modificada de Cot/tpl-2 induce un cambio en el proteoma de células Hela en las que un aumento de 5,3 veces en la expresión de COX-2 se presenta como el único cambio significativo.

Existen numerosos estudios que establecen el papel de Cot/tpl-2 truncada en tumorogénesis.

Nosotros hemos demostrado que Cot/tpl-2 regula la invasión celular modulando varios de los pasos implicados en este proceso. Así, nuestros resultados indican que en células que sobreexpresan la forma truncada de Cot/tpl-2, la capacidad invasiva de las células presenta un aumento estadísticamente significativo de 1,5 veces con respecto a las células control. Aún más, en células en las que la expresión de Cot/tpl-2 se encuentra bloqueada mediante un ARN silenciador (ARNsi) específico, la capacidad invasiva de las células se reduce a niveles inferiores a los presentados por las células control (3% de invasión en células transfectadas con los ARNsi de Cot/tpl-2 frente al 31% que presentan las células transfectadas con el vector vacío).

También demostramos en este trabajo que la capacidad de Cot/tpl-2 truncada de inducir la migración celular se debe en parte a la capacidad de Cot/tpl-2 para inducir la expresión de COX-2.

Además la sobreexpresión de Cot/tpl-2 implica una disminución de 2 a 4 veces en los niveles de adhesión celular y un incremento de 1,8 veces en la migración celular con respecto a células control. El ARNsi de Cot/tpl-2 produce por el contrario una disminución en los niveles de migración celular (4 veces).

Cot/tpl-2 también regula la reorganización de los componentes del citoesqueleto, así las células que sobreexpresan Cot/tpl-2 truncada presentan características comunes con células invasivas como una disminución en el número de fibras de estrés y una polarización de los microtúbulos.

En consonancia con estos datos, las células en las que la expresión de Cot/tpl-2 se encuentra bloqueada presentan un mayor número de fibras de estrés, un menor número de complejos ARP 2/3 y una mayor tinción para paxilina lo que concuerda con células con baja capacidad migratoria/invasiva.

Se ha descrito que una cierta activación basal de Rho es necesaria para la producción de la fuerza adhesiva requerida para inducir la motilidad celular y como un exceso de activación de Rho inhibe la migración celular. Corroborando estos datos las células transfectadas con el ARNsi de Cot/tpl-2 presentan mayores niveles de la forma activa de Rho (Rho-GTP).

Todos estos datos sirven para ayudar a esclarecer el papel de Cot/tpl-2, tradicionalmente relacionada con fenómenos de inmunidad innata, en los fénomenos de invasión y migración celular. Esto refuerza la idea de que los mecanismos de invasión celular e inflamación comparten bases moleculares comunes.

En la citada publicación el doctorando ha contribuido al diseño y realización de los experimentos cuyos resultados se ven plasmados en las figuras 2, 3, 5, 6 y 7. Se puede considerar que su grado de aportación al proyecto es elevado como refleja el hecho de que el doctorando esté considerado como primer autor del manuscrito.

Expression of Cot/tpl-2 MAP kinase kinase kinase influences the invasive potential of transformed cells.

Running title: The levels of Cot regulate cell invasion.

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Cot/tpl-2, a MKKK with a role in innate immunity, was identified in a truncated/modified form as an oncogene, Cot T. We demonstrate here that Cot regulates different steps in the process of cell invasion. Cells that overexpress Cot T exhibit a faster rate of invasion than control cells, while the expression of Cot siRNAs diminishes the rate of cell invasion. Overexpression of Cot T induces a 2- to 4-fold decrease in cell adhesion and a 1.8-fold increase in the migration of these cells with respect to control cells, in part mediated by the induction of COX-2 protein expression. Furthermore, the blockage of endogenous Cot expression reduces cell migration by 4-fold with respect to control cells, probably due to the increase in the accumulation of Rho-GTP in these cells. Cot also regulates the reorganization of the cytoskeleton with cells overexpressing Cot T showing a loss of actin stress fibres and a polarization of the microtubule cytoskeleton, characteristics of invasive cells. Accordingly, Cot siRNAs expression depletes cells of Arp2/3 complexes and augments the staining for paxillin and the formation of stress fibres. Inflammatory and invasive process share some common molecular events and accordingly, we show here that Cot is involved in cell invasion in addition to its established role in innate immunity.

INTRODUCTION

Cot/tpl-2 is one of the main MKKKs that activates the MKK1-Erk1/Erk2 pathway in response to the extracellular signals involved in innate immunity. The importance of this kinase is demonstrated by the tpl-2 knock-out mice which is resistant to LPS/D-Galactosamine-induced pathology due to the poor production of TNF-α. In fact, in tpl-2 knock-out macrophages LPS is no longer able to activate Erk1/Erk2 (Dumitru, Ceci et al. 2000). Stimulation of macrophages with CpG-DNA also induces Cot activity (Sugimoto, Ohata et al. 2004). However, while Cot is the sole MKKK1 that activates Erk1/Erk2 upon LPS stimulation (Dumitru, Ceci et al. 2000; Eliopoulos, Dumitru et al. 2002; Caivano, Rodriguez et al. 2003; Waterfield, Zhang et al. 2003), MKKK1 proteins other than Cot up-regulate Erk1/Erk2 in response to CpG-DNA stimulation

(Sugimoto, Ohata et al. 2004). Other stimuli also activate Cot in other cell types and indeed, in anti-CD40- or TNFα-stimulated B cells, as well as in IL-1-stimulated HeLa cells, Cot is the sole MKKK that activates the MKK1-Erk1/Erk2 pathway (Eliopoulos, Wang et al. 2003; Rodriguez, Pozo et al. 2006).At present, the mechanism by which Cot becomes fully active after cell stimulation is not completely understood (Stafford, Morrice et al. 2006). However, irrespective of the cell type and the stimulus, it is accepted that Cot forms a stable and inactive complex with p105 NF-κB and other proteins in resting cells. Extracellular signals that activate Cot first induce the activation of the IKK complex. Activated IKKβ phosphorylates p105 NF-κB, which is then rapidly degraded by the proteasome pathway to p50 NF-κB, a subunit of the NF-κB transcription factor. Consequently, Cot is released from the complex and it then becomes capable of transducing the stimulatory signal (Waterfield, Zhang et al. 2003; Beinke, Robinson et al. 2004; Waterfield, Jin et al. 2004; Stafford, Morrice et al. 2006). In macrophages or B cells, endogenous Cot is essential for the activation of Erk1/Erk2 by LPS or TNFαstimulation, however it does not play a role in the activation of JNK and p38γ(Dumitru, Ceci et al. 2000). Moreover, in HeLa and MEF cells stimulated by IL-1, Cot also activates Erk1/Erk2 but not p38α and JNK (Das, Cho et al. 2005; Rodriguez, Pozo et al. 2006). However, in tpl-2-/- MEF cells the activation of Erk1/Erk2, JNK, and NF-κB is defective following stimulation with TNF□. Therefore, it has been proposed that signalling through endogenous Cot is both stimulus and cell type specific (Das, Cho et al. 2005).

The abnormal enhancement of Cot activity that follows overexpression of wt Cot or of the oncogenic truncated/modified Cot (Cot T), induces activation of the Erk1/Erk2, JNK, p38γ, and ERK5 MAP kinase pathways (Patriotis, Makris et al. 1994; Salmeron, Ahmad et al. 1996; Hagemann, Troppmair et al. 1999; Lin, Cunningham et al. 1999; Chiariello, Marinissen et al. 2000). Overexpression of Cot also induces the up-regulation of transcription factors such as AP-1, NFAT, and NF-κB, in part as a consequence of the stimulation of the different MAP kinase pathways (Ballester, Velasco et al. 1998; Tsatsanis, Patriotis et al. 1998; Tsatsanis, Patriotis et al. 1998; Belich, Salmeron et al. 1999; Hagemann, Troppmair et al. 1999; Lin, Cunningham et al. 1999; Chiariello, Marinissen et al. 2000; de Gregorio, Iniguez et al. 2001; Sebald, Mattioli et al. 2004). Moreover, the activation of the Erk1/Erk2 pathway by Cot links Cot activity with the up-regulation of CREB (Eliopoulos, Dumitru et al. 2002) and E2F (Velasco-Sampayo and Alemany 2001).

The human Cot gene was first identified as an oncogene with a 3´ rearrangement that leads to the expression of a truncated/modified protein, Cot T (Miyoshi, Higashi et al. 1991). The first 397 amino acids of Cot T and the wt Cot proto-oncogene are identical. However, the COOH-terminal 69 amino acids in wt Cot contain a sequence that targets the protein for proteasome degradation (Gandara, Lopez et al. 2003), and they are replaced by 18 non-homologous amino acids in Cot T (Miyoshi, Higashi et al. 1991; Ceci, Patriotis et al. 1997), unmasking the transforming capacity of the protein (Ceci, Patriotis et al. 1997; Gandara, Lopez et al. 2003). Interestingly, overexpression of wt Cot is also capable of conferring a transformed phenotype on established cell lines (Chan, Chedid et al. 1993; Chiariello, Marinissen et al. 2000; Gandara, Lopez et al. 2003). The rat homologue of the human Cot gene, tpl-2, was also identified in a similar truncated/modified form due to its capacity to induce T cell transformation (Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993). Moreover, the insertion of the Mouse Mammary Tumour Virus (MMTV) into the last intron of the tpl-2 gene is associated with transformation of mammary gland cells (Erny, Peli et al. 1996). Indeed, a modification in the 3´ region of the Cot gene was more recently confirmed in some human adenocarcinoma tumors (Clark, Reynolds et al. 2004). Furthermore, other modifications in the Cot/tpl-2 gene have been shown to be associated with cancer progression. Thus, the locus of the human Cot gene is amplified in some human breast cancers without any modification of the coding sequence of the Cot gene (Sourvinos, Tsatsanis et al. 1999). Indeed, a correlation between high Cot mRNA levels and human large granular lymphocyte disorder has also been proposed (Christoforidou, Papadaki et al. 2004). Moreover, infection of the human T cell leukemia virus type-I (HTLV-I) appears to induce the expression of an unusually stable N-terminal truncated Cot protein (Babu, Waterfield et al. 2006). Abnormally high levels of Cot protein are also commonly associated with Epstein Barr virus malignancies, such as nasopharyngeal carcinoma and gastric cancer (Eliopoulos, Davies et al. 2002). All these data indicate that, although physiologically wt Cot is involved in innate and adaptive immunity, mutations in the Cot gene results in the expression of a protein linked with cell malignancies.

In this paper we demonstrate that Cot regulates cell invasion by modulating different steps involved in this process. Oncogenic Cot T expression increases cell invasion and migration, and reduces cell adhesion. Moreover, we also provide evidence that the blockage of endogenous Cot expression, traditionally involved in innate immunity, diminishes cell invasion and migration. Furthermore, we conclude that Cot modulates cell migration by modulating Rho activity, COX-2 expression, and by inducing changes in cytoskeletal organization.

MATERIALS ANS METHODS

Cell culture, cell transfection and stimulation — HeLa cells were maintained in an atmosphere of 95 % air and 5 % CO_2, in DMEM supplemented with: 10 % (v/v) of heated-inactivated foetal bovine serum (FBS, Hy-Clone); 100 U/ml penicillin; and 100 μg/ml gentamicin. The day before stimulation, the cells were plated at a density of $8x10^6$ cells/100 mm dish and they were maintained in the same medium but with the concentration of FBS reduced to 0.1 % (v/v). After 24 h in this medium, the cells were stimulated with 40 ng/ml of IL-1 alpha (Roche) for the times specified in the figure legends. HeLa cells were transfected using lipofectamin with the empty pclx vector (30 μg), pclx Cot T (30 μg), the empty pSR vector (30 μg), or with a mixture of the pSR 368 (15 μg) plus pSR 525 (15 μg) Cot siRNA plasmids described previously (Rodriguez, Pozo et al. 2006). The transfection of any of these constructs endows the transfected cells with resistance to puromycin. After rinsing twice in PBS, transfected cells were selected for 24 h after transfection by incubating them for 24-30 h in 3 % (v/v) FBS/DMEM, with 100 U/ml penicillin, 100 μg/ml gentamicin and 1 μg/ml puromycin (Gibco-BRL). As a control, non-transfected cells were also exposed to puromycin. The selected transfected cells were then used in different assays.

Western blot analysis and luciferease assays— After 24 h in the presence of 1 μg/ml puromycin, the medium was removed and the cells were incubated for another 24 h in 0.1 % (v/v) FBS/DMEM plus antibiotics. Before performing western blots, the cells were rinsed twice with ice cold PBS and the attached cells were then were solubilized in cold lysis buffer: 50 mM Tris acetate (pH 7.0); 1 mM EDTA; 1 mM EGTA; 1 % (w/v) Triton X-100; 1 mM sodium orthovanadate; 10 mM sodium glycerophosphate; 50 mM NaF; 5 mM sodium pyrophosphate; 0.27 M sucrose; 0.1 % (v/v) 2-merchaptoethanol; and a complete proteinase inhibitor mixture (1 tablet/50 ml). The lysates were then snap frozen in liquid nitrogen and stored in aliquots at -70ºC until they were analyzed. Cell extracts were thawed at 0ºC, centrifuged for 10 min at 24,000xg to pellet the debris, and the protein concentration was determined by the Bradford method (Bio-Rad). Western blots were performed using 35 μg of protein from HeLa transfected cells, as described previously (Rodriguez, Pozo et al. 2006). PVDF membranes were probed with anti-Cot (Calbiochem or Santa Cruz), anti-P-JNK, anti-P-Erk1/Erk2, anti-P-p38α (Cell Signalling) or anti-COX-2 (Transduction Laboratories) antibodies. Membranes were also probed with anti-Erk-

2 (Cell Signalling) or anti-PDI (Gandara, Lopez et al. 2003), as a control of protein loading. Antibody binding was visualized using the chemiluminescent method (ECL, Amersham-Pharmacia- Biotech). To measure luciferase activity, HeLa cells ($5x10^5$) were transfected in 60 mm-plates with the empty pclx vector (3.6 μg) or with pclx Cot T (3.6 μg) together with 1.3 of μg p3x-Nf-κB Luc (de Gregorio, Iniguez et al. 2001). The cell medium was replaced 18 h after transfection with DMEM/FBS 0.1 % (v/v) and 24 h later, luciferase activity was measured as indicated previously (Ballester, Tobena et al. 1997).

DIGE and mass spectrometry analysis — Cells were lysed using 150 μl of DIGE lysis buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4 % CHAPS and protease inhibitors) and they were mixed for 15 min at 4ºC. To perform the assay, 50 μl of the solubilized extracts were precipitated using the 2D-Clean Up Kit (Amersham) and resuspended in 50 μl of DIGE lysis buffer. The protein concentration was determined by the Bradford method (Bio-Rad). Subsequently, 75 μg of Cot T or siRNAs cell extracts were mixed with 75 μg of control cell extract and loaded by the cup loading method in a strip (3-11 pH range 24 cm) previously rehydrated in: Urea 7 M; Thiourea 2 M; CHAPS 2 % (w/v); DTE 10 mM; IPG buffer pH 3-10; and bromophenol blue. The strips were focused at 0.05 mA/IPG strip in the Ettam IPGphor II System (Amersham) using the following increasing voltage steps: 150 V for 1 h; 500 V 1 h; linear gradient to 1000V over 2 h; linear gradient to 4000 V within 8 h; and 8000 V until steady state. After IEF, the strips were equilibrated for 12 min in 6 M Urea, 100 mM Tris, 30 % Glycerol, 2 % SDS and 0.5 % DTT, and then for 5 min with 6 M Urea, 100 mM Tris, 30 % Glycerol, 2 % SDS and 4.5 % Iodoacetamide. Finally, the proteins were separated on 12 % SDS-Tris-glycine gels. Gels were scanned in a Typhoon 9400 scanner (GE Healthcare) at the appropriate wavelengths using filters for the Cy2, Cy3, and Cy5 dyes. Mass spectrometry analysis of the protein spots of interest was performed by manually excising the spots from micro preparative gels (500 μg of cell extract protein) using biopsy punches. The proteins selected for analysis were reduced in-gel, alkylated and digested with trypsin as described previously (Shevchenko, Chernushevic et al. 2002). Briefly, the spots were washed twice with water, shrunk for 15 min with 100 % acetonitrile and dried in a Savant SpeedVac for 30 min. The samples were then reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56ºC and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, the samples were digested with 12.5 ng/μl

sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37ºC. After digestion, the supernatant was collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of α -cyano-4-hydroxy-transcinnamic acid matrix (3 mg/ml, Sigma) in 50 % acetonitrile was added to the dried peptide digest spots and again allowed to air-dry at room temperature. MALDI-TOF MS analyses were performed in a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) at the Genomics and Proteomics Center (Complutense University of Madrid) operating in positive reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin. The MALDI-TOF/TOF mass spectrometry analysis produces peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular weights. For protein identification, the non-redundant NCBI database was searched using MASCOT 1.9 (matrixscience.com) through the Global Protein Server v3.5 from Applied Biosystems. The search parameters used were: Carbamidomethyl Cysteine as fixed modification and oxidized methionine as a variable modification; peptide mass tolerance was 50-80 ppm; and 1 missed trypsin cleavage site. In the protein identification, the probability scores were greater than the score fixed as significant with a p-value less than 0.05.

MTT assay — HeLa cells ($40x10^3$) transfected with the different constructs described above were counted utilizing the MTT reduction colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, ROCHE), as indicated by the manufacturer´s instructions. After puromycin selection, the cells were incubated for 20 h in DMEM containing 1 % or 3 % (v/v) FBS/DMEM, or 50 % (v/v) conditioned medium (the medium resulting from incubating NIH3T3 cells in serum-free DMEM for 48 h)/DMEM. Afterwards, MTT (5 mg/ml) was added and 4 h later the tetrazolium salt was diluted with solubilisation buffer and incubated overnight at 37ºC. Subsequently, the absorbance was measured in a colorimetric reader at 590 nm. As a control of the number of cells added to the different 96 wells, another set of the same transfected cells were plated for 3 h, to allow the cells to attach to the bottom of the wells. After this period of time MTT was added and 4 h later, the cells were solubilized and the absorbance measured as described above.

Invasion assay. **—** Cell invasion assays were performed as described previously (Jones, Royall et al. 1997) with some minor modifications. Matrigel transmigration assays were performed using cell culture inserts (8 μm pore size, Beckton and Dickinson) placed in 24 well tissue culture plates. The lower side of the insert was coated with 10 μg/ml of fibronectin for 1 h. Diluted 1:10 matrigel (1 mg/ml, BD Biosciences) in serum free medium/DMEM was added to the upper side of the insert and incubated for 2 h at 37ºC to gel. After the 24 h puromycin selection, the different transfected HeLa cells were harvested by trypsin/EDTA treatment and washed twice with PBS. Cells were then resuspended in DMEM before 105 cells/well were added in 500 μl medium and left to adhere to the upper side of the insert. The lower chamber contained 1 ml of 50 % (v/v) conditioned medium/DMEM, as a chemoattractant. Cells were incubated for 24 h at 37°C in a 5 % CO2 incubator and subsequently, unwanted cells on the upper or lower surface of the filter were completely removed by wiping with a cotton swab. The filters were then fixed in cold acetone and stained with haematoxylin/eosin. Cells that had invaded the Matrigel and reached the lower surface of the filter, as well as cells on the top, were counted under a light microscope at a magnification of ×200. To determine the percentage invasion, cells from 10 fields were counted and the percentage invasion was calculated as: Σ cells of the bottom / ∑(cells of top + cells of bottom) x100.

Adhesion assay **—** The 96 well plates were covered with fibronectin (10 μg/ml, Sigma-Aldrich), vitronectin (0.3 μg/ml, Sigma-Aldrich), laminin (20 μg/ml, Sigma-Aldrich), or gelatin (10 μg/ml, Sigma-Aldrich) overnight at 4ºC, and then washed with PBS. The different transfected and selected HeLa cells, were incubated for 20 h in DMEM plus 1 % or 3 % (v/v) FBS, or 50 % (v/v) conditioned medium and they were labelled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, SIGMA) following the manufacturer's instructions. The cells were then resuspended in DMEM at a concentration of 5x105 cells/ml. Subsequently, 100 μl of the different cell suspensions was added to each well and the HeLa cells were allowed to adhere to the different matrix proteins for 45 min at 37ºC in an atmosphere of 95 % air, 5 % CO2, before removing non-adherent cells by washing in PBS. Adherent HeLa cells were quantified in a spectrofluorometer at excitation and emission wavelengths of respectively 500 nm and 530 nm.

Quantitative reverse transcription PCR assay— Untransfected HeLa cells, or those transfected with the plasmids indicated above, were stimulated with IL-1 (40 ng/ml) for different periods of time. The RNA from these cells was then isolated and reverse transcribed as described previously (Ballester, Velasco et al. 1998). Quantitative PCR was performed and the expression of MMP1, MMP2, MMP9, MMP10, MMP11, and 18 S was analysed using specific probes for real time PCR (Applied Biosystems) according to the manufacturer's instruction.

Wound healing assay— HeLa cells transfected with pclx, pSR, pclx Cot T, or with pSR 525 plus pSR 368 were plated at a confluency (105 cells per well) in 96 multiwell plates pre-coated with Matrigel (1 mg/ml). Wounds were made using a p10 tip, the cells were washed twice with PBS to remove cellular debris, and the medium was changed to DMEM with 1 % or 3 % FBS (v/v). The cell cultures were left to recover for 24 h in a 37ºC, 5 % CO2 incubator. Photographs were taken at different times and cell migration was determined as the difference in μm in the wound width between 0 h and 16 h utilizing the Act 2U Nikon measurement tool following the manusfacturer´s instructions. In some assays and as indicated in the figure legend, NS398 (Sigma, 50 μM) Y27632 (Sigma, 20 μM) or PGE2 (Sigma, 5 mM) were also added, 3 h, 20 min or 10 min before wounding and these compounds were then maintained throughout the assay. The viability of the different transfected cells, with or without the different inhibitors, was studied by performing trypan blue staining. A similar number of viable cells (over 95 %) was obtained in all the different conditions.

Immunocytochemical studies of cytoskeletal components in HeLa cells— Puromycin selected HeLa cells were cultured on eight-well glass slides (Lab-Tec, Chamber slide system, Nalgene Nunc International) in complete medium overnight and then depleted in 0.1 % (v/v) FBS/DMEM for 16-24 h. Subsequently, cells were fixed with 2 % (v/v) paraformaldehyde/H2O and permeabilized with 0.5 % (v/v) triton/H2O. The cells were then incubated overnight at 4ºC with DAPI (1 μg/ml, Invitrogen), phalloidin-FITC (1:200, SIGMA), or with the antibodies against: β-tubulin (1:200, SIGMA); vimentin (1:200, Dako Cytomation); paxillin (1:200, Becton and Dickinson); ARP3 (1:100, Becton and Dickinson). The glass slides were then washed with PBS and incubated with the secondary ALEXA 594 antibody (Molecular Probes) for 1 h at RT. After washing with PBS, the slides were coverslipped with 90 % buffered glycerine. Photomicrography was performed using an OLYMPUS microscope with a Nikon digital camera.

Measurement of Rho-GTP bound levels in Hela cells— Puromycin selected HeLa cells transfected with pclx, pSR, pclx Cot T, or with pSR 525 plus pSR 368 were plated at 50-70% confluency (1.5x106 cells) in a 60mm dish. The cells were starved for 20 h in 0.1% (v/v) FBS/DMEM. After starving, the cells were washed once in ice-cold PBS, solubilized in 100 μl of G-lisa lysis buffer plus inhibitors (Cytoskeleton) and centrifuged at 24000g for 4 min to remove cellular debris. The protein concentration was determined using the G-Lisa protein determination reagent (Cytoskeleton) and 25 μg of protein extracts were then used to test the Rho A/B/C -GTP levels using the G-lisa Rho A/B/C colorimetric method and measured as optical absorbance at 405

RESULTS

Cot regulates cell invasion— We have recently shown that Cot is the sole MKKK regulating the activation of the Erk1/Erk2 pathway in response to IL-1 stimulation in HeLa cells (Rodriguez, Pozo et al. 2006). Thus, utilizing DIGE technology we investigated whether Cot activity modulates the proteome of HeLa cells. HeLa cells were transfected with a combination of two different Cot pSR siRNAs (pSR 368 plus pSR 525) to block endogenous Cot expression, as well as with the empty pSR vector. The positive puromycin selected HeLa cells were further incubated for 24 h in 0.1 % (v/v) FBS/DMEM and to assay endogenous Cot expression, cell extracts were subjected to western blot analysis. In cells co-transfected with the two Cot pSR siRNA plasmids endogenous Cot expression was blocked, and there was also less phospho Erk1/Erk2 than in cells transfected with the empty vector (Fig. 1A and (Rodriguez, Pozo et al. 2006)). To increase Cot activity, HeLa cells were transfected with the pclx Cot T plasmid or with the empty pclx vector and the surviving puromycin resistant cells were incubated for 24 h in 0.1 % (v/v) FBS/DMEM. Subsequently, the different cell extracts were again subjected to western blot analysis to determine Cot T expression as well as the phosphorylation state of different MAP kinases such as phospho-Erk1/Erk2, phospho-JNK and phospho-p38γ. As described previously, overexpression of Cot T was correlated with an increase in the phosphorylation of Erk1/Erk2 and JNK (Fig. 1B and (Salmeron, Ahmad et al. 1996; Chiariello, Marinissen et al. 2000)). Likewise, cells co-transfected with the pclx Cot T and p3x-Nf-κB Luc constructs exhibited 25-fold higher Luc activity than cells co-transfected with the empty pclx plasmid and the p3x-Nf-κB Luc construct (Fig. 1B and (Tsatsanis, Patriotis et al. 1998; Belich, Salmeron et al. 1999; Lin, Cunningham et al. 1999)). Together, these data indicate that by transfecting cells with Cot siRNA or Cot T plasmids we can modulate the intracellular expression of Cot and consequently, the signal transduction pathways regulated by this kinase.

The DIGE proteome analysis of positive selected HeLa cells did not identify any consistent or statistically significant change in the protein expression pattern between control and pSR368/pSR525 Cot siRNAs transfected HeLa cells. However, the analysis of the different 2D gel images obtained from the cell extracts of pclx Cot T and pclx transfected HeLa cells showed that the expression of one spot was consistently and significantly induced in Cot T overexpressing cells. Mass spectrometry analysis allowed us to identify the corresponding protein as COX-2 which was present in 5.3 fold greater quantities in Cot T cell extracts than control extracts (Fig. 1C).

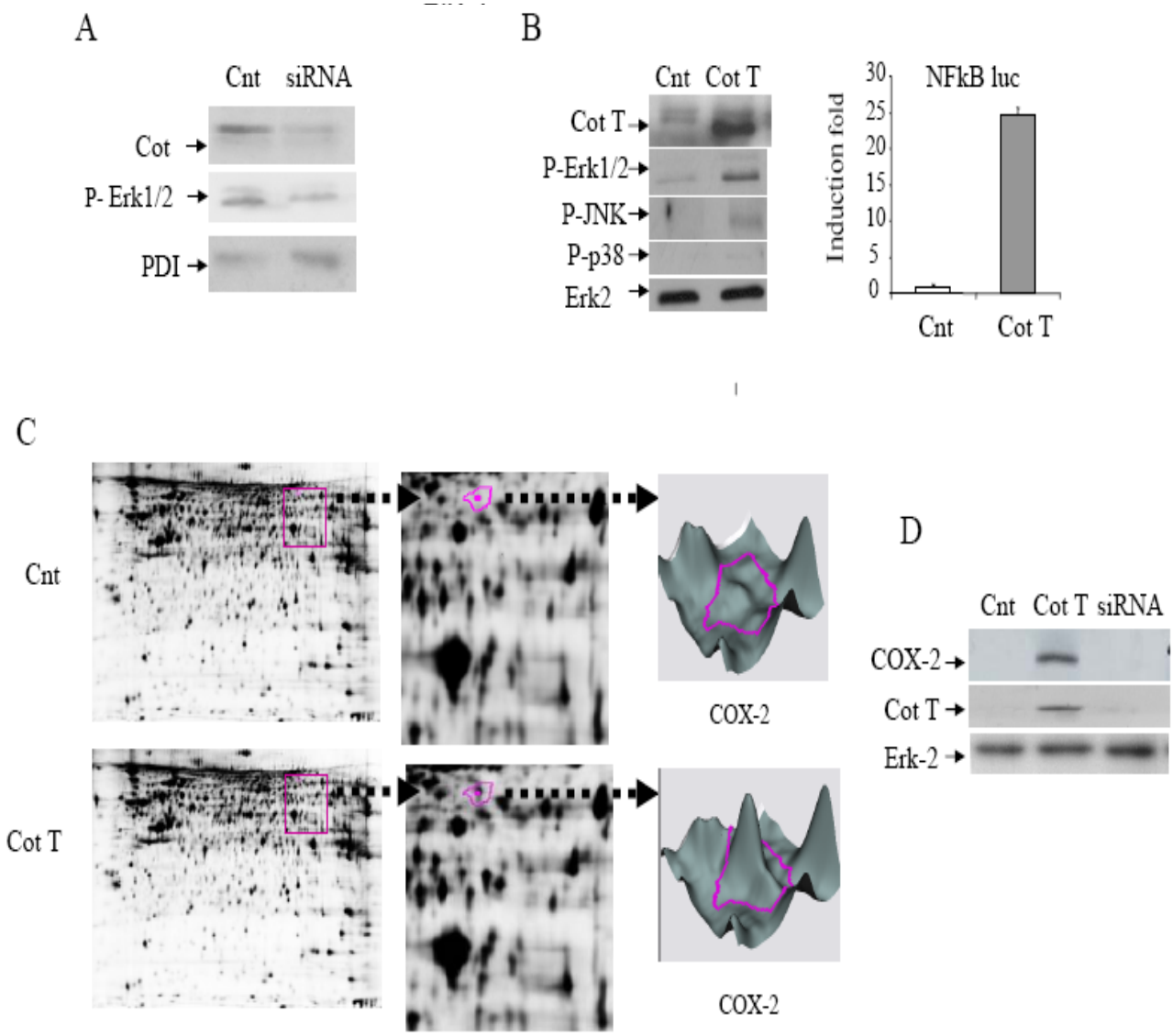


Figure 1. Regulation of different signal transduction pathways and COX-2 expression by Cot in HeLa cells. (**A**) HeLa cells transfected with pSR (Cnt) or with a mixture of pSR 368 plus pSR 525 Cot siRNA (siRNA), were puromycin selected and incubated for a further period of 24 h in 0.1% (v/v) FBS/DMEM medium. The cell lysates were subjected to western blot analysis and membranes were probed with anti-Cot (Santa Cruz), anti-phospho-Erk1/2, and anti-PDI antibodies. Antibody binding was revealed using a chemiluminiscent method (ECL, Amersham-Pharmacia-Biotech). (**B**) HeLa cells were transfected with pclx (Cnt), or with pclx Cot T (Cot T) and treated as described in (A). Membranes were probed with anti-Cot (Calbiochem) and anti-P-Erk1/Erk2, anti-Erk2, anti-P-p38α MAPK, and anti-P-JNK antibodies. Luciferase activity values of HeLa cells co-transfected with pclx (Cnt) or with pclx Cot T (Cot T) together with the p3x-Nf-κB Luc construct represent the means +/- S.D. of three determinations performed in triplicate. (**C**). 2-D DIGE images of the 2D gel containing extracts of control (Cnt) and Cot T expressing cells labelled with Cy3 and Cy5. The images of the COX-2 spot are also shown. (**D**) Western blot analysis of cells extracts of HeLa cells transfected with pclx (Cnt), pclx Cot T (Cot T), or with a mixture of pSR 368 plus pSR 525 Cot siRNA (siRNA), puromycin selected, and incubated for a further period of 24 h in 0.1% (v/v) FBS/DMEM medium. Membranes were probed with anti- COX-2, anti-Cot (Calbiochem), and anti-Erk2. (**A, B, D**) Western blot figures show one representative experiment of the three performed.

As expected, western blot analysis confirmed that Cot T cell extracts contained higher levels of COX-2 protein than control or pSR368/pSR525 Cot siRNAs cell extracts. Furthermore, the induction of COX-2 protein expression by overexpressing Cot T was observed independent of the medium in which the cells were mantained (DMEM plus 1 % or 3 % (v/v) FBS or 50 % (v/v) conditioned medium; data not shown). Mechanistic studies indicated that COX-2 is involved in tumour cell invasiveness and migration, in this way contributing to its effects on tumorigenesis. Moreover, a causal link between epithelial tumorigenesis and COX-2 has also been shown in genetically manipulated animal models, in which the appearance of invasive and malignant tumours was greatly exaggerated and secondary metastases were observed (Kirschenbaum, Liu et al. 2001; Nithipatikom, Isbell et al. 2002; Sinicrope and Gill 2004; Singh, Berry et al. 2005; Hiraga, Myoui et al. 2006; Larkins, Nowell et al. 2006).

Considering the accepted role of COX-2 in tumorigenesis, we studied how the differences in the expression of the Cot protein might affect HeLa cell proliferation. Accordingly, positively selected pclx, pSR, pclx Cot T, or pSR368/pSR525 Cot siRNAs expressing HeLa cells were incubated in DMEM plus 1 % or 3 % FBS (v/v), or conditioned medium 50 % (v/v), and subjected to a MTT cytotoxic cell proliferation assay. No significant differences in cell viability were observed between the different transfected HeLa cells, irrespective of the incubation media utilized (Fig. 2A). To study the possible role of Cot in cell invasiveness, we therefore investigated the ability of the different transfected HeLa cells to invade an extracellular matrix. The behaviour of selected pclx, pSR, pclx Cot T, or pSR368/pSR525 HeLa transfected cells was evaluated in a Matrigel gel invasion chamber assay. Systematically, cells expressing Cot T exhibited the highest rates of invasion and 45+/-7 % of cells transmigrated through the membrane pores emerging on the lower surface of the membrane. In contrast, 31+/-2 % of the cells transfected with the empty pclx vector appeared on the bottom of the chamber (Fig. 2B). As expected, untransfected cells or cells transfected with the empty pSR vector exhibited the same transmigration rate as cells transfected with the empty pclx vector (data not shown). Furthermore, cells transfected with pSR368/pSR525 Cot siRNAs that have diminished endogenous Cot levels, exhibited the lowest invasive capacity, only 10 +/- 3 % of transmigrated cells (Fig. 2B). The levels of Cot also influenced the invasive capacity of the human breast adenocarcinoma MCF-7 cells (data not shown). Since cell invasion is considered as multi-step phenomena, we studied the role of Cot in each of these different processes.

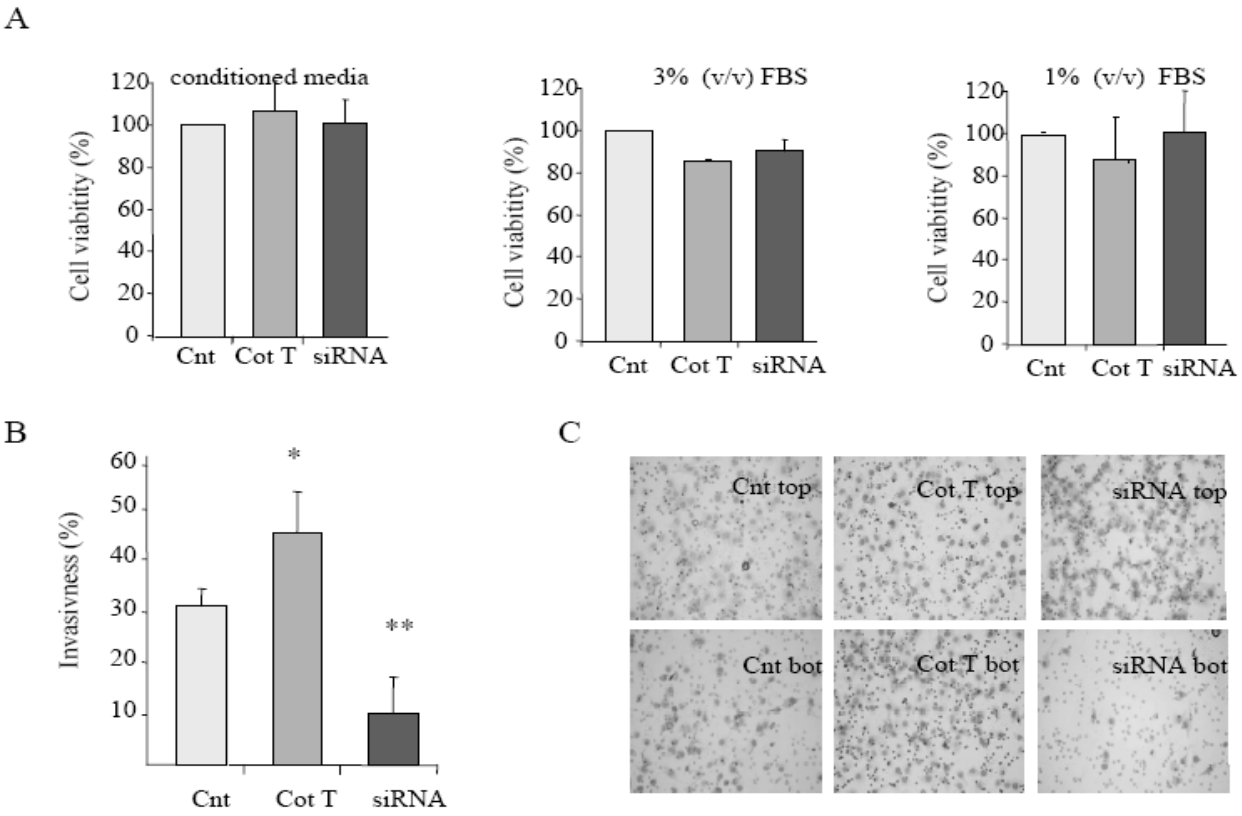


Figure 2. Cot regulates cell invasion. (A) MTT assay of Hela pclx, pSR, pclx Cot, or of pSR 368 plus pSR 525 Cot siRNA transfected cells, puromycin selected, and incubated for 24 h in the following extracellular media, 3% (v/v) FBS/DMEM, 1% (v/v) FBS/DMEM or 50% (v/v) conditioned medium/DMEM. Figures show the means +/- S.D. of three independent experiments performed with pclx (Cnt), pclx Cot (Cot T), or pSR 368/pSR 525 Cot siRNA (siRNA) transfected cells. 100% is given to control value. T-Student test was carried out to determine the difference between control and Cot T and Cot siRNA transfected cells (*P<0.05, **P<0.01, ***P<0.001). Similar results were obtained with pSR and pclx empty vectors transfectecd cells or non transfected cells **(B, C)** HeLa cells were transfected with pclx, pSR pclx Cot T, or pSR 368h plus pSR 525h Cot siRNA. After 24h of transfection, cells were selectioned with puromicin 1 μg/ml for 24 h and $5X10^4$ cells were replated in fibronectin-matrigel precoated 0,8 μg pore cell culture inserts. Cells were allowed to invade for 24 h in conditioned medium and then stained and fixed. Cell invasion was determined counting cells of the top and the bottom of at least 10 fields of 200x magnification cell inserts photographs. Pannel **B** shows the media +/- S.D in % of invasive cells respect to the total cells, of 6 experiments performed in duplicate cell of pclx (Cnt), pclx Cot T (Cot T), and pSR 368h/ pSR 525h Cot siRNA (siRNA) transfected HeLa cells. Pannel **C** shows photographs of one of the 10 representative areas per insert counted to determine the % of migration per sample and assay.

Cot is involved in HeLa cell migration—. To further establish the specific role of Cot in invasion, we determined whether Cot influences cell migration. Accordingly, we carried out a wound healing assay on positive selected HeLa cells transfected with pclx, pSR, pclx Cot T, as well as those transfected with pSR368/pSR525 Cot siRNAs.

The assay was performed in DMEM medium containing 1 % or 3 % (v/v) FBS and in both circumstances, overexpression of Cot T augmented cell migration and faster wound healing was observed. The percentage migration of Cot T overexpressing cells was 58+/-7 % in 3 % (v/v) FBS and 63+/-19 % in 1 % (v/v) FBS, while cells transfected with the empty pclx vector displayed a migration rate of 37+/-9 % in 3 % (v/v) FBS or 36+/-14 % in 1 % (v/v) FBS (Fig. 3A). A similar migration rate was observed with untransfected cells or in those transfected with pSR (data not shown). More interestingly, the blockage of endogenous Cot expression diminished cell migration under all the conditions tested. While the ability of the pSR368/pSR525 Cot siRNAs transfected HeLa cells to migrate was reduced to a 11+/-5.2 % in 3 % (v/v) FBS, the percentage of migration was reduced even further to 8+/-5 % when the migration assay was performed in the presence of 1 % (v/v) FBS (Fig. 3A).

We then studied whether the enhanced expression of COX-2 induced by the overexpression of Cot T in HeLa cells (Fig. 1B) plays indeed a role in the increased migration capacity of Cot T overexpressing cells. Wound healing assays were performed in the presence of the specific COX-2 inhibitor NS398 (50 μM), which impaired the migration of Cot T transfected cells by about 70 % (Fig. 3C). NS398 (50 μM) also induced a small decrease in the capacity of control HeLa cells to migrate**.** Recent evidence suggests that COX-2 induced carcinogenesis is mediated by PGE_2, one of the products derived from COX-2 activity (Han and Wu 2005; Han, Michalopoulos et al. 2006; Xu, Han et al. 2006). To further confirm the role of COX-2 in the induction of HeLa cells migration, we performed the wound healing assays of Cot T and control transfected cells in the presence of 5 mM PGE_2. The migration of control cells increased in the presence of PGE_2, the cells migrating approximately twice as fast and displaying a similar migration capacity as Cot T overexpressing cells (Fig. 3C)**.** On the other hand, the addition of PGE_2 did not further increased the capacity of cells transfected with Cot T to migrate in the wound healing assay (Fig. 3C), further evidence that the enhanced migration when Cot T is overexpressed is mediated by COX-2 expression. However, the addition of PGE_2 to the pSR368/pSR525 Cot siRNAs transfected HeLa cell in the migration assays did not increase the capacity of these cells to migrate to the levels observed with Cot T overexpressing cells (Fig. 3C).. Hence, endogenous Cot appears to regulate cell migration by controlling additional events than the ones that PGE_2 can regulate.

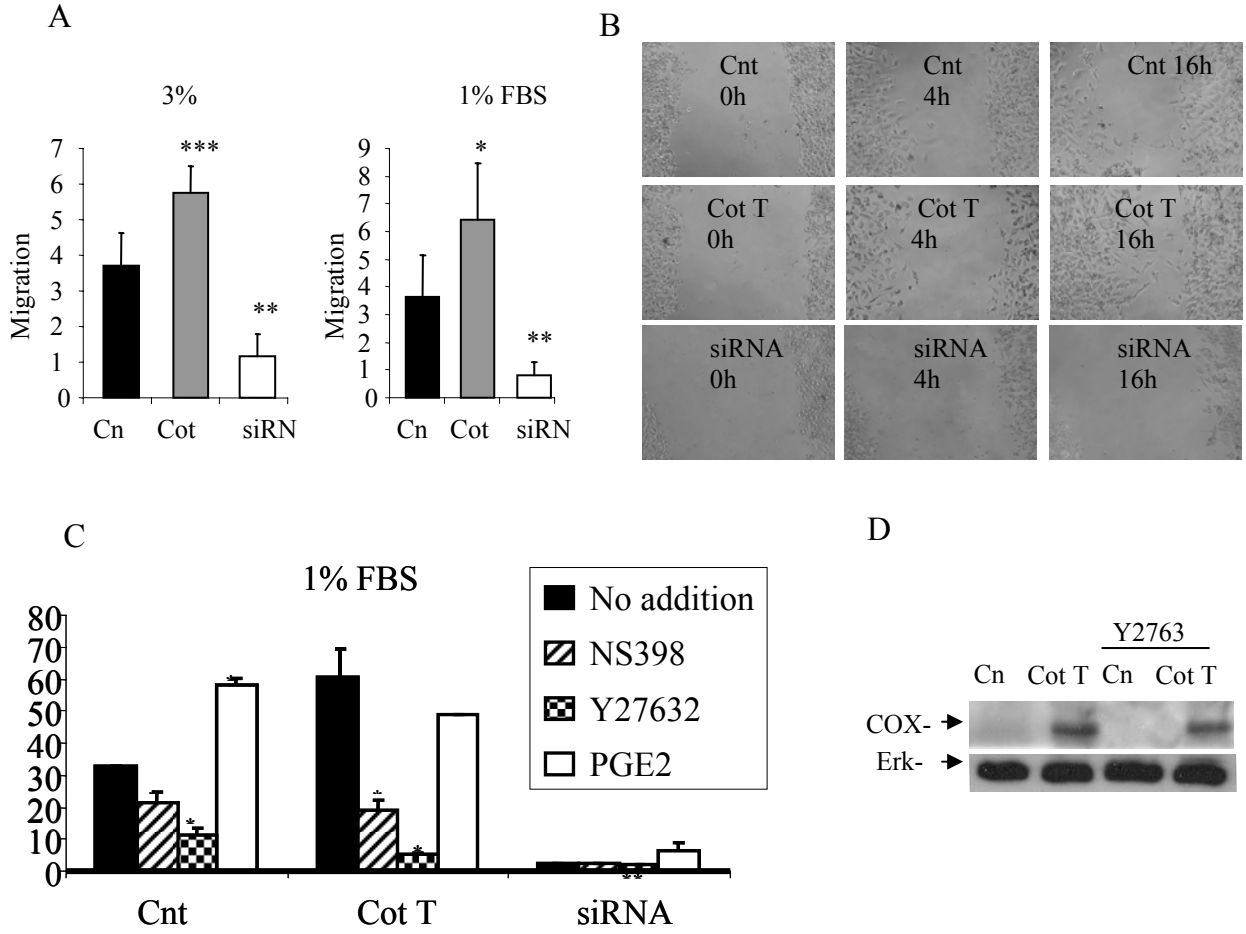


Figure 3. Cot regulates cell migration. HeLa pclx, pSR, pclx Cot, or pSR 368 plus pSR 525 Cot siRNA transfected cells, puromicyn selected, and incubated for 24 h in 3 % (v/v) FBS/DMEM or in 1 % (v/v) FBS/DMEM extracellular media were subjected to cell migration assays. Pannel **A** shows the figures of the means +/- S.D. of three independent experiments performed in triplicate of pclx (Cnt), pclx Cot (Cot T), or pSR 368 plus pSR 525 Cot siRNA (siRNA) transfected cells. Wound repair is expressed as a percentage of the initial wound area after 16 h. T-Student test was carried out to determine the difference between the different samples (*P<0.05, **P<0.01, ***P<0.001). Similar results were obtained with pSR and pclx empty vectors transfectecd cells. **B** shows one representative experiment performed in 1 % (v/v) FBS/DMEM medium. **C** Wound repair analysis performed with HeLa pclx, pSR, pclx Cot, or pSR 368 plus pSR 525 Cot siRNA transfected cells as indicated in A and performed in 1 % (v/v) FBS/DMEM in the presence or absence of 50 μM of NS398 (COX-2 inhibitor) or 20 μM of Y27632 (ROCK inhibitor) or 5 μM PGE_2. The different drugs were added before the scraching to the incubation media, as described under Material and Methods. Wound was performed using a 10 μl pipette tip and cells were allowed to migrate for 24h. The drugs were maintained in the cell culture medium during the stimulation. The experiment shows the means +/- S.D. of three experiments performed separately by triplicate. (**D**) Western blot figures show one representative experiment of the three performed with extracts of HeLa cells transfected with pclx (Cnt), pclx Cot T (Cot T), puromycin selected, and incubated for a further period of 24 h in 1 % (v/v) FBS/DMEM medium in the presence or absence of 50 μM of NS398. Membranes were probed with anti- COX-2, anti-Cot (Calbiochem), and anti-Erk2.

ROCK has been implicated in the increased cell migration observed during tumour cell invasion and metastasis of such cells and can be inhibited by the ROCK inhibitor Y-27632 (Itoh, Yoshioka et al. 1999; Imamura, Mukai et al. 2000; Gutjahr, Rossy et al. 2005). The presence of Y-27632 (20

μM) in the wound healing assays performed with the control, Cot T overexpressing, or Cot siRNAs transfected Hela cells reduced the migratory capacity by 2.9-, 11.2-, and 1.1- fold, respectively (Fig. 3C). These data indicate that the induction of COX-2 expression by Cot T did not overcome the influence of ROCK on the migration of these transfected cells. To exclude the possibility that the ROCK inhibitor impaired cell migration by blocking the expression of COX-2, the expression of COX-2 was analysed in western blots of extracts from control and Cot T transfected cells preincubated for 24 h with Y-27632 (20 μM). However, similar levels of COX-2 expression were detected irrespective of whether the cells had been exposed to the ROCK inhibitor (Fig. 3D).

Cot T overexpression reduces cell adhesion— To further investigate whether Cot regulates cell migration and invasion through mechanisms that involve changes in cell adhesion, we carried out cell adhesion assays in presence of different extracellular matrix cell components. Hence, we analyzed the cell adhesion capacity of pclx, pSR, pclx Cot T, as well as pSR368/pSR525 Cot siRNAs transfected HeLa cells in culture dishes coated with vitronectin, fibronectin, laminin, or gelatin. In the first instance, we determined the adhesive capacity of the different transfected cells that had been maintained for 20 h in DMEM with 50 % (v/v) conditioned medium. Irrespective of the nature of extracellular substrate, the expression of Cot T resulted in a 2-4 fold decrease in the number of adherent cells when compared to control cells (Fig. 4A). On the other hand, the inhibition of endogenous Cot expression did not seem to affect the adhesive capacity of the cells. The pSR368/pSR525 Cot siRNAs transfected cells only exhibited a significant increase in their adhesion to fibronectin when compared to control cells (1.4 fold). Since laminin and fibronectin proved to be the best substrates for the attachment of HeLa cells, we determined whether the capacity of Cot T overexpressing cells to adhere to these substrates also diminished when cells were preincubated for the last 20 h in medum containing1 % or 3 % (v/v) FBS. However, in these conditions the capacity of Cot T transfected cells to adhere to fibronectin or laminin was also significantly impaired (Fig. 4B).

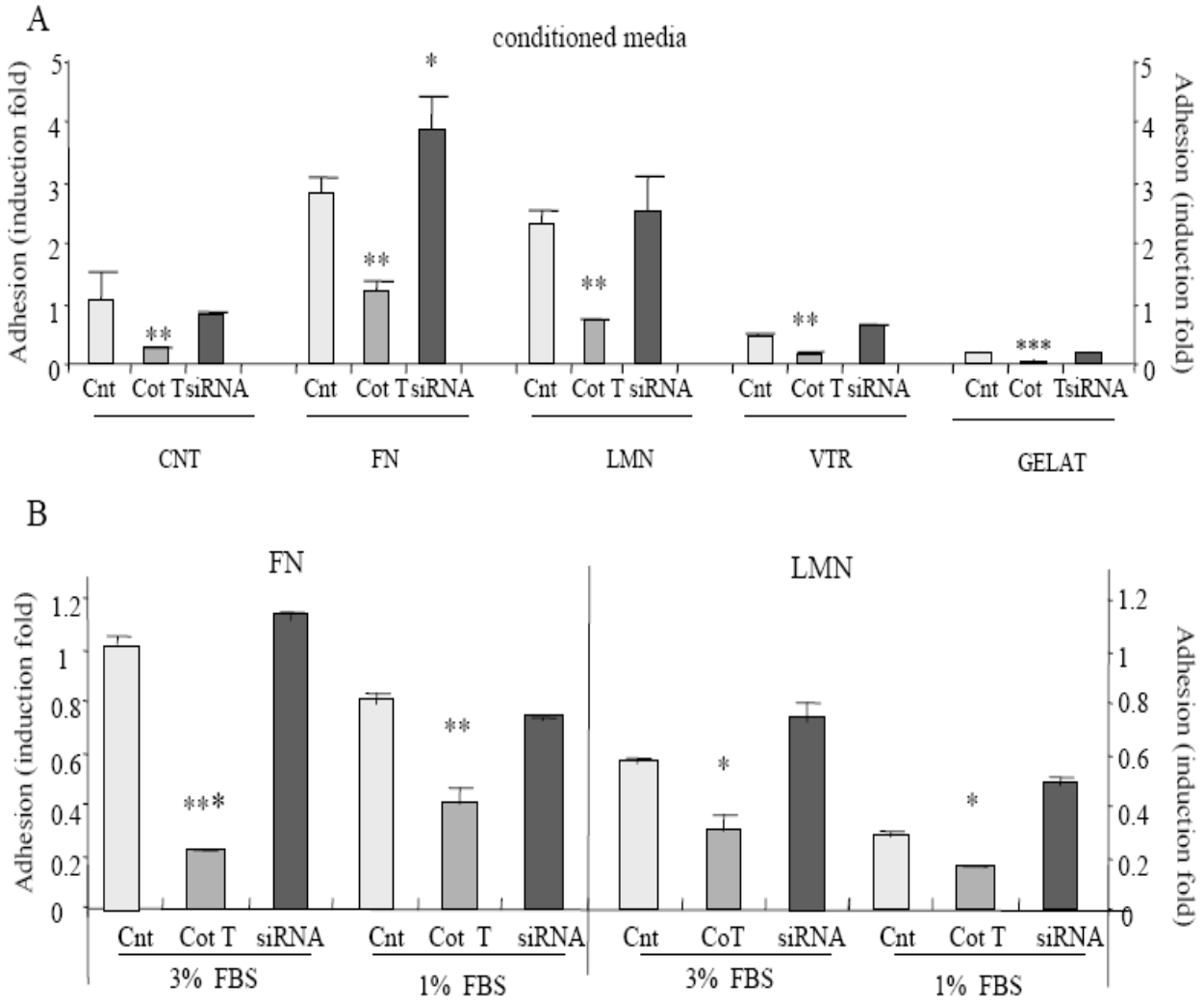


Figure 4. Overexpression of Cot T decreases cell adhesion. **(A)** The adhesion to fibronectin (10 μg/ml, FN), vitronectin (0.3 μg/ml, VTR), laminin (20 μg/m, LMNl) or gelatin (10 μg/ml, GELAT) in 96 well plates, of pclx, pSR, pclx Cot, or pSR 368 plus pSR 525 Cot siRNA transfected HeLa transfected cells, and incubated for the last 24 h in 50 % (v/v) conditioned/DMEM medium, was tested. The figures show the means +/- S.D. of three independent experiments performed in triplicated of pclx (Cnt), pclx Cot (Cot T), or pSR 368 plus pSR 525 Cot siRNA (siRNA) transfected cells. T-Student test was carried out to determine the difference between the different samples (*P<0.05, **P<0.01, ***P<0.001). Similar results were obtained with pSR and pclx empty vectors transfectecd cells. (**B**) Pclx, pclx Cot, or pSR 368 plus pSR 525 Cot siRNA transfected HeLa transfected cells were cultured the last 24 h in 3 % (v/v) FBS/DMEM, or in 1 % (v/v) FBS/DMEM media and adhesion assays to fibronectin (10 μg/ml) and laminin (20 μg/ml) were carried. The significance of the differences in cell adhesion of three independent assays performed in triplicate was statistically demonstrated by T-Student test.

Regulation of MMP1, MMP9, MMP10, and MMP11 mRNA levels by endogenous Cot activity— Proteolytic cleavage of the extracellular matrix is an essential step in many physiological and pathological processes including tumour cell invasion. This cleavage requires the activity of several key enzymes named metalloproteinases (MMPs). The constitutive level of MMP gene expression is normally low although their expression can up-regulated by many different extracellular factors. In fact, increased expression of MMPs is observed in many disease states, including neoplasia, arthritis, and other inflammatory processes (Chakraborti, Mandal et al. 2003; Arribas 2005, Mancini, 2006 #280; Deryugina and Quigley 2006; Fingleton 2006). Accordingly, we used quantitative RT-PCR to measure the mRNA levels of MMP1, MMP9, MMP10, and MMP11 in HeLa cells transfected with the pSR, pSR368/pSR525, pSR, pclx, or pclx Cot T plasmids and incubated for 24 h in the presence of 0.1 % (v/v) FBS. The cells transfected with pSR368/pSR525 showed slightly weaker expression of the different MMP mRNAs when compared to the levels expressed in control cells. However HeLa cells expressing Cot T exhibited an increase in MMP10 and MMP1 mRNA of 240+/-117 and 1663+/-276 fold, respectively (mean of 3 experiments performed in triplicate). Considering the elevated expression of MMPs in pathological inflammatory processes (Okamoto, Akuta et al. 2004) and that Cot mediates the activation of Erk1/Erk2 in response to the proinflammatory cytokine IL-1 (Rodriguez, Pozo et al. 2006), we investigated whether endogenous Cot mediates the increase in MMP mRNA expression induced by IL-1 in HeLa cells. Maximal expression of MMP9 and MMP11 mRNA was observed 1-2 h after IL-1 stimulation, while maximal levels of MMP1 and MMP10 mRNA were observed after 4-6 h (Fig. 5A). The induction of these MMPs by IL-1 was repressed by exposing the cells to the MKK1 inhibitor U0126, a specific inhibitor of the Erk1/Erk2 pathway (data not shown). In accordance with this data, the inhibition of Cot expression by the transfection of the pSR368/pSR525 Cot siRNAs completely abolished the induction of MMP1, MMP9, MMP10, and MMP11 mRNA expression, following IL-1 stimulation over the times evaluated (Fig. 5B).

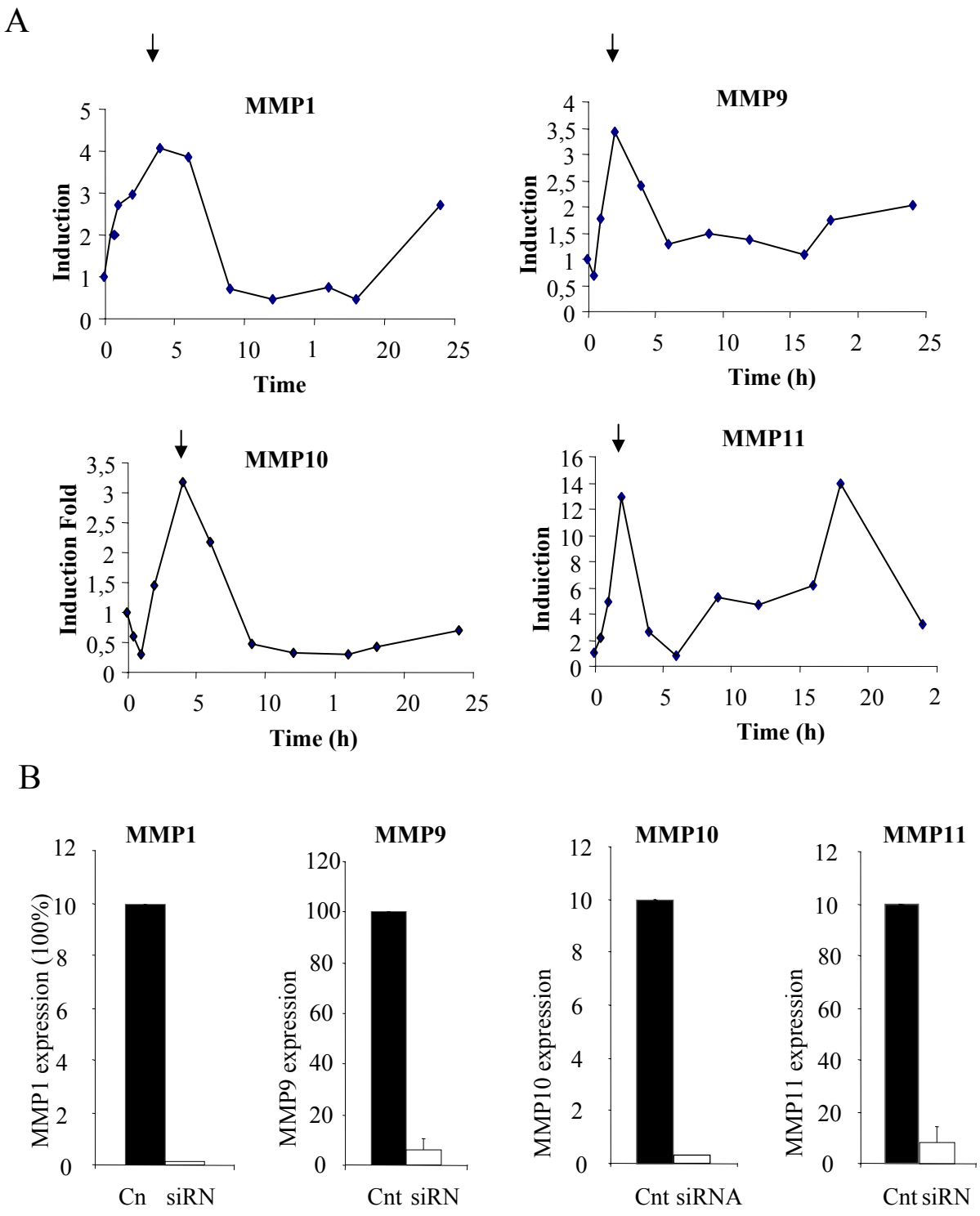


Figure 5. Cot regulates MMP1, MMP9 MMP10, and MMP11 mRNA expression. (**A**), HeLa cells, incubated the last 24 h in 0.1 % (v/v) FBS/DMEM, were stimulated with IL-1 (40 ng/ml) for the following times 0.5, 1, 2, 4, 6, 9, 12, 16, 18, and 24 h. The MMP1, MMP9, MMP10, and MMP11 mRNA levels were determined by RT-PCR analysis. The figure shows a representative experiment of the three performed for each MMP. **(B)** HeLa cells transfected with pSR or with pSR638/pSR525 Cot siRNA and resistant to puromycin were stimulated with IL-1 (40 ng/ml) for 2 h to determine MMP9 and MMP11 mRNA levels or for 4 h to determine the MMP10 and MMP11 mRNA levels. The graph shows the means +/- S.D. of three experiments performed in duplicate and 100 % of MMP expression is given to the mRNA levels obtained in IL-1 stimulated pSR transfected HeLa cells.

Cot modulates cytoskeleton reorganisation—. Cell migration, a key event in cell invasion, requires different processes to be completed including the formation and extension of lamellipodia, and their subsequently attachment to different extracellular matrix components. Finally, the retraction of pseudopods at the leading edge of the cells and the formation of new structures related to attachment (such as mature focal adhesions) complete this highly dynamic process. Reorganization of the actin, through polymerization and depolymerization of actin microfilaments or the formation of stress fibres, is essential to induce attachment to the extracellular matrix (Yamazaki, Kurisu et al. 2005). Therefore we determined whether Cot modulates stress fibres formation in HeLa cells transfected with empty pclx, pSR vectors, pclx Cot T, as well as with pSR368/pSR525 Cot siRNAs and plated onto fibronectin-coated coverlips for 16 h. On this substrate, Cot T overexpressing cells displayed fewer actin stress fibres whereas pSR368/pSR525 Cot siRNAs cells developed abundant stress fibres (Fig. 6A, left panel, and 6B). It has been proposed that the distribution of actin is mainly responsible for determining cell shape and the direction of cell movement, in conjunction with vimentin intermediate filaments and microtubules. Microtubules and intermediate filaments are involved in the maintenance of cellular shape and cell motility, highlighting the importance of the tubulin and vimentin based cytoeskeletal reorganization in cell migration (Vasiliev 2004; Honore, Pasquier et al. 2005). In fact, microtubules are essential for the cell-extracellular matrix interaction, participating in the polymerization of actin at the edge of the cell (Small, Kaverina et al. 1999; Waterman-Storer, Worthylake et al. 1999; Small, Geiger et al. 2002). In pSR368/pSR525 Cot siRNAs and control transfected HeLa cells, microtubules were localized in a perinuclear ring-like structure, a characteristic of attached and non-migratory cells (Fig. 6A middle panel). However, when Cot T was overexpressed this circular microtubule organization was no longer apparent and the microtubules adopted a polarized aspect throughout the cytoplasm (Fig. 6A middle panel). This microtubule structure induces cell elongation and allows to the ends of microtubules to approximate to the plasma membrane, where they can stimulate lamellipodia formation (Small, Kaverina et al. 1999; Waterman-Storer, Worthylake et al. 1999; Small, Geiger et al. 2002; Watanabe, Noritake et al. 2005). Moreover, indirect immunofluorescence to visualise the distribution of intermediate filaments indicated that the overexpression of Cot T also induced the polarization of the vimentin structures. In contrast, pSR368/pSR525 Cot siRNAs transfected HeLa cells displayed a disorganized pattern of intermediate filaments in which vimentin was localized to the perinuclear area (Fig. 6B right panel). All these data demonstrate that Cot T overexpressing

cells presented morphological characteristics of transformed poorly spreading cells, with a reduced peripheral cytoplasm, fusiform morphology and a decrease in the number of stress fibres.

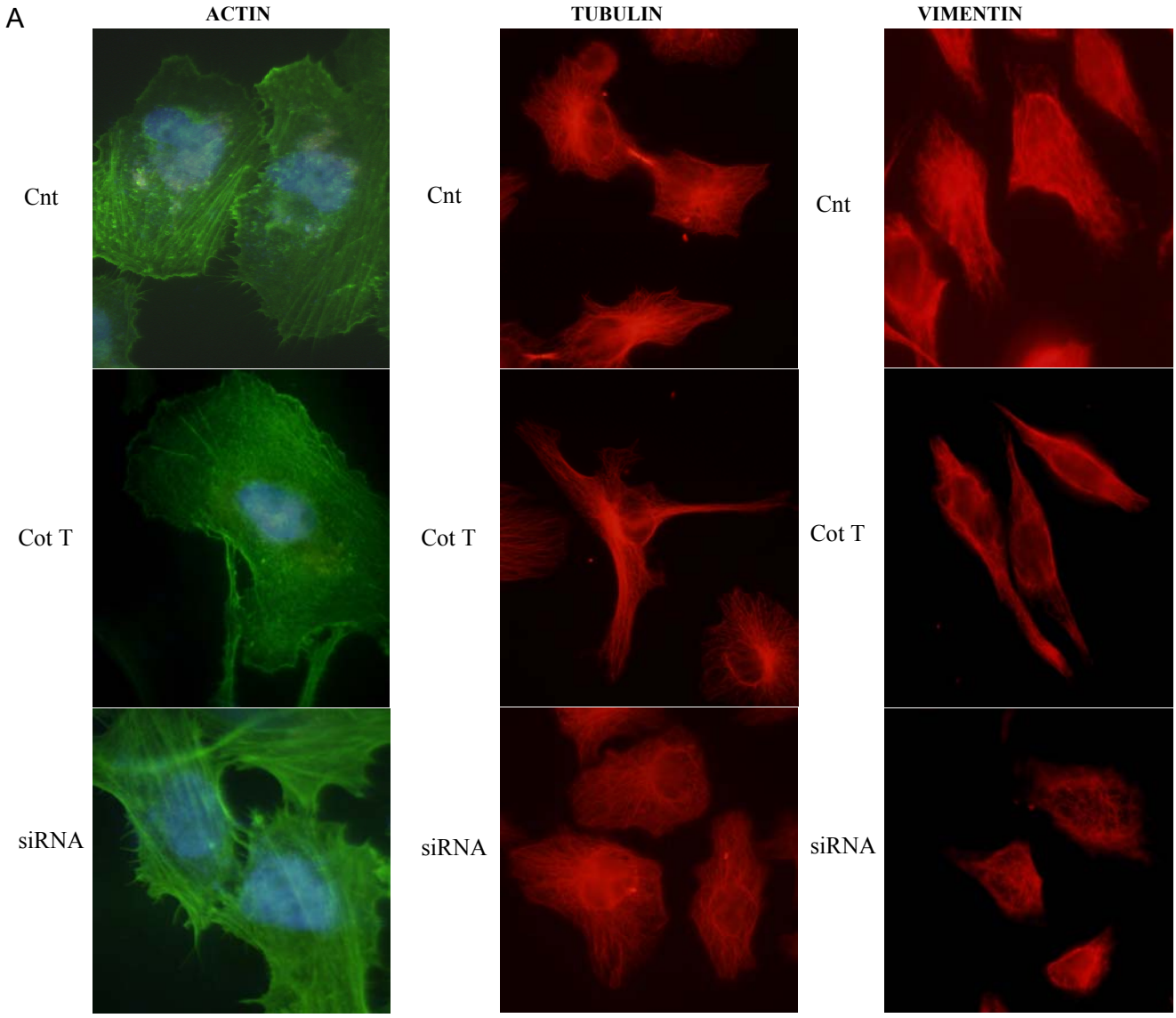
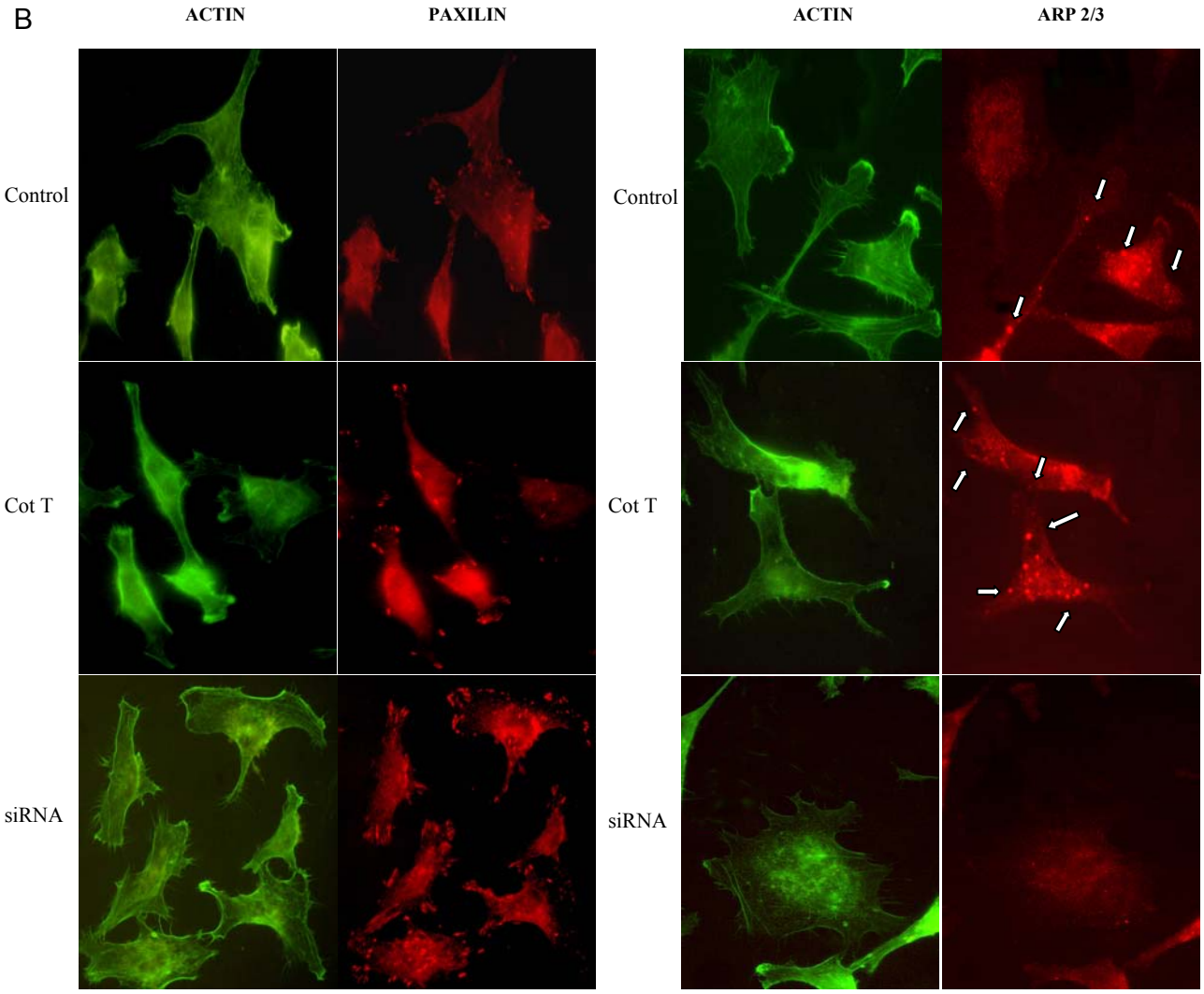


Figure 6. Cot induces cytoesqueleton reorganization. HeLa cells ($5x10^5$) transfected with pclx, pclx Cot T or pSR 368 plus pSR 525 Cot siRNAs were puromycin selected, plated in 10 μg/ml of fibronectin pre-coated coverslip and cultured for 16 h in 0.1 % (v/v) FBS/DMEM medium. Cells were stained and analyzed by fluorescence microscopy. (**A)** Composite images of actin (green) and DAPI (blue) is shown. Actin staining was performed using phalloidin-FITC (1:200) together with DAPI (1μg/ml) to stain nucleus, tubulin and vimentin staining was performed using respectively anti-tubulin (1:200) or anti-vimentin monoclonal antibodies (1:200) and Alexa-594 (1:2000, red) as a secondary antibody. (**A, B)** Photographs were taken at 63x with a Olimpus fluorescence microscopy coupled to a Nikkon digital camera. Similar results have been obtained with three different set of transfected cells.

We also examined the localization of the focal adhesion protein paxillin, since localization of paxillin at theses sites is concomitant with the ability to form strong focal adhesions (Hagel, George et al. 2002; Zaidel-Bar, Ballestrem et al. 2003). Paxillin immunostaining demonstrated

that the recruitment of paxillin to focal adhesions increased in pSR368/pSR525 Cot siRNAs transfected cells when compared to control and Cot T transfected cells (Fig. 6B left panel).

In contrast, during the formation of lamellipoda, the construction of new microfilaments requires the prior formation of a protein complex at the leading edge of the cells. This complex, is essential to initiate actin polymerization and it includes several proteins, such as ARP 2/3 (Machesky, Reeves et al. 1997; Pollard and Borisy 2003).



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Cot siRNAs transfected cells these ARP3 containing complexes were hardly detected (Fig. 6B right).

The Rho family of small GTPases regulates many facets of cytoskeletal organization (Nobes and Hall 1999; Schwartz and Shattil 2000; Worthylake and Burridge 2003). To evaluate the possibility that the Cot induced reorganization of the cytoskeleton is mediated by Rho, HeLa cells were transfected with pclx, pSR, pclx Cot T, as well as with pSR368/pSR525 Cot siRNAs, and incubated for 24 h in the presence of 0.1 % (v/v) FBS/DMEM. When measured in the different cells extracts the overexpression of Cot T in HeLa cells did not affect the levels of Rho-GTP. However Cot siRNAs expression in cells augmented the levels of Rho-GTP to 1.64+/- 0.21 fold that observed in control cells, indicating that endogenous Cot regulates Rho-GTP levels. Moreover, this increase in the Rho-GTP in Cot siRNAs cells could not be blocked by prior exposure to 5 mM PGE_2 (Fig. 7).

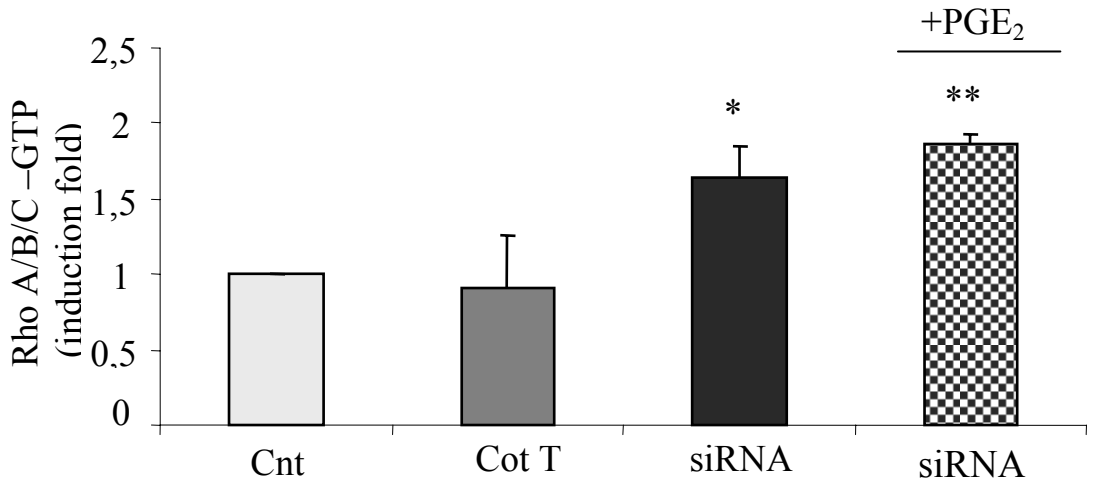

Figure 7. Rho-GTP bound levels are increased in Cot siRNA cells.
Hela cells transfected with empty vector, Cot T, or 368 plus 525h siRNA plasmids were selectioned for 24h with 1 mg/ml of puromicin. Selectioned cells were starved for 20h in 0.1% FBS media and then 368/525h cells were stimulated with 5 μM of PGE_2 for 5 min. After incubation cells were collected and assayed for Rho A/B/C according to the manufacturer´s instructions. The panel shows the mean +/- S.D. of three experiments performed separately by duplicate. T-Student test was carried out to determine the difference between the different samples (*P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

Experiments performed with the Cot/tpl-2 knock-out mouse as well as with Cot knock-down cells have indicated that endogenous Cot is involved in innate and adaptative immunity (Dumitru, Ceci et al. 2000; Eliopoulos, Dumitru et al. 2002; Caivano, Rodriguez et al. 2003; Eliopoulos, Wang et al. 2003; Waterfield, Zhang et al. 2003; Sugimoto, Ohata et al. 2004; Das, Cho et al. 2005; Rodriguez, Pozo et al. 2006). Here we show that endogenous Cot also regulates cell invasion and migration. The human Cot gene was actually discovered as an oncogene in a truncated/modified form Cot T (Miyoshi, Higashi et al. 1991), initially linking this protein with tumorigenesis. Moreover, the murine homologue, tpl-2, was also identified in a similar truncated/modified form and it plays an essential role in the progression of T lymphomas (Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993). In this context, overexpression of Cot in a T cell line increases COX-2 promoter transcription by activating the NFAT response elements in the human COX-2 promoter (de Gregorio, Iniguez et al. 2001). In LPS-stimulated macrophages, endogenous Cot regulates COX-2 expression by modulating Erk1/Erk2 activity (Eliopoulos, Dumitru et al. 2002). Although the mechanism by which Cot activity increases COX-2 expression in HeLa cells remains to be established, it is accepted that activation of Erk1/Erk2 and activation of the NFκB transcription factor are essential to increase COX-2 mRNA levels in this carcinoma line as well as in very close related cell lines (Chun and Surh 2004; Wu 2005; Telliez, Furman et al. 2006). Indeed, overexpression of Cot T in HeLa cells augments the activity of both pathways. Here we show that the 5.3 fold increase in the expression of COX-2 protein is the only consistent proteomic change in HeLa cells as a consequence of Cot T overexpression. In this context, it should be noted that in Epstein-Barr virus associated malignancies Cot activity is deregulated and it triggers the expression of COX-2 (Eliopoulos, Davies et al. 2002).

In recent years, the role of COX-2 in tumorigenesis has become more clearly established (Sinicrope and Gill 2004; Singh, Berry et al. 2005; Hiraga, Myoui et al. 2006; Larkins, Nowell et al. 2006). Here, we show that the enhanced migration of cells induced by Cot T overexpression is at least partially mediated by COX-2. However, the fact that the impaired migration of siRNAs Cot transfected cells can not be recovered by the addition of PGE_2 indicates that endogenous Cot controls other events that are not regulated by this prostaglandin. In fact, the addition of PGE_2 to Cot siRNAs cells also failed to reverse the increased levels of Rho-GTP observed in these cells. Moreover the addition of PGE_2 to HeLa cells did not increase the phosphorylation of Erk1/Erk2

(data not shown) and the low steady state phospho-Erk1/Erk2 levels exhibit by siRNAs Cot cells, could be one reason why their migration is impaired. Indeed, the activation of Erk1/Erk2 pathways plays an essential role in cell migration and invasion (Reddy, Nabha et al. 2003; Huang, Jacobson et al. 2004; Ehrenreiter, Piazzolla et al. 2005; Rajalingam, Wunder et al. 2005). Moreover, the addition of a specific Erk1/Erk2 inhibitor to Cot T expressing and control cells blocked cell migration (data not shown). Together, these data indicate that the capacity of Cot to regulate the Erk1/Erk2 pathway is critical for the migration of these cells.

Our data indicate that endogenous Cot is not likely to be involved in cell adhesion. However, the overexpression of Cot T reduces the adhesion of HeLa cells to all the extracellular matrix components tested. The transcription of the plasminogen activator inhibitor-1 (PAI-1) gene is induced by an increase in the activity of Erk1/Erk2 and JNK (Reddy, Nabha et al. 2003; Providence and Higgins 2004; Buchwalter, Gross et al. 2005) and cells overexpressing Cot T exhibited higher levels of PAI-1 protein than control or siRNAs cells (data not shown). Considering that PAI-1 reduces the ability of cells to attach to different types of extracellular matrices (Czekay, Aertgeerts et al. 2003), the modulation of cell adhesion by overexpressing Cot T could be mediated by the up-regulation of PAI-1 protein expression.

MMPs play a crucial role in tumour invasion and in fact, MMP1, MMP9, MMP10, and MMP11, whose mRNA expression is induced by endogenous Cot, are reported to participate in cell malignancy (Pulyaeva, Bueno et al. 1997; John and Tuszynski 2001; P, Rhys-Evans et al. 2001; Tolboom, Pieterman et al. 2002; Fridman, Toth et al. 2003; Mercapide, Lopez De Cicco et al. 2003; Thorns, Walter et al. 2003; Kousidou, Roussidis et al. 2004; Sato, Takino et al. 2005; Sossey-Alaoui, Ranalli et al. 2005; Turpeenniemi-Hujanen 2005; Yuan 2005) . However activation of different signal transduction pathways are required to increase the expression of these 4 MMP transcripts, since overexpression of Cot T up-regulates the mRNA levels of MMP1 and MMP10 but it does not alter the transcription of the MMP9 and MMP11 genes.

Cell migration requires cytoskeleton reorganization and we show that Cot modulates the organization of three cytoskeletal components, actin microfilaments, microtubule, as well as intermediate filaments. Indeed the morphological changes observed in Cot T cells, including the loss of actin stress fibres and the polarization of microtubules, are characteristic of oncogenically transformed cells (Riento and Ridley 2003). These features are therefore consistent with the higher invasion capacity of cells overexpressing Cot T On the other hand the morphological changes observed in Cot siRNAs cells, include the appearance of abundant stress fibers, intense staining for paxillin indicative of the formation of strong focal adhesions (Hagel, George et al. 2002;

Zaidel-Bar, Ballestrem et al. 2003), and the virtual absence of Arp2/3 complexes indicative of the formation of few lamellipodia (Machesky, Reeves et al. 1997; Pollard and Borisy 2003). These changes are characteristic of cells that migrate poorly.

Rho GTPases also play a central role in the motile responses involving the actin cytoskeleton and/or microtubule network. ROCK is an important effector that mediates the formation of stress fibres and focal adhesion contacts induced by activated Rho (Riento, Guasch et al. 2003; Riento and Ridley 2003). Our data indicate that ROCK activity is indeed required to induce cell migration in the different transfected HeLa cells, irrespective of the levels of Cot expression. On the other hand, although basal Rho activity is required for the generation of adhesive forces, excessive Rho activity inhibits migration probably through the formation of strong focal adhesions (Ridley 2001; Arthur WT 2001) . This is in accordance with the induction of stress fibres in Cot siRNAs cells, as well as the increase in the Rho–GTP bound form and the low migratory capacity of these cells.

During the last 15 years evidence has accumulated that mutations in the Cot gene results in the expression of a protein linked with cell malignancy (Miyoshi, Higashi et al. 1991; Chan, Chedid et al. 1993; Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993; Erny, Peli et al. 1996; Ceci, Patriotis et al. 1997; Sourvinos, Tsatsanis et al. 1999; Chiariello, Marinissen et al. 2000; Eliopoulos, Davies et al. 2002; Gandara, Lopez et al. 2003; Clark, Reynolds et al. 2004; Christoforidou, Papadaki et al. 2004; Babu, Waterfield et al. 2006). In this paper we demonstrate that an increase in Cot activity induces invasive properties in transformed cells by regulating different steps involved in this process. It is becoming clear that inflammatory and invasiveness processes share certain common molecular elements (Chakraborti, Mandal et al. 2003; Karin 2006; Aggarwal R 2006) . Here we show that endogenous Cot, which has an established role in innate adaptive processes, also regulates cell invasion.

Acknowledgements

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DISCUSIÓN

Durante el desarrollo de esta Tesis Doctoral hemos establecido que existen al menos dos señales extracelulares, el LPS y la IL-1, capaces de inducir una activación de Cot/tpl-2.

Cot/tpl-2, es una MAP quinasa quinasa quinasa (MKKK) que regula fisiológicamente, y a través de MKK1, la activación de la MAPK ERK1/ERK2 (Salmeron, Ahmad et al. 1996; Caivano y Cohen 2000; Dumitru, Ceci et al. 2000; Rodriguez, Pozo et al. 2006).

La ruta de ERK1/ERK2 es susceptible de ser activada, además de por Cot/tpl-2, por otras MKKKs entre las que se encuentran RAF y Mos (Troppmair, Bruder et al. 1994; Pham 1995; Xu S 1995; Salmeron, Ahmad et al. 1996).

Sin embargo, desde el punto de vista fisiológico, y a diferencia de RAF y Mos, Cot/tpl-2 es la única MKKK capaz de activar, en ciertas condiciones específicas, otras rutas de señalización como NF-κB y JNK que, en fibroblastos, se produce en respuesta al TNF-α (Das, Cho et al. 2005).

Además, la naturaleza de los ligandos extracelulares que activan a RAF y Cot/tpl-2 es distinta. La actividad de RAF se incrementa por la unión de ligandos a receptores con actividad tirosina quinasa (Troppmair, Bruder et al. 1994; Avruch y 2001) mientras que Cot/tpl-2 se activa por la unión de ligandos a receptores tipo IL-1/Toll-like y por la unión de ligandos a ciertos receptores de muerte.

Se ha descrito que los receptores de LPS (TLR4) e IL-1 (IL-1R) comparten homología estructural en su región citoplasmática, en la que presentan un dominio denominado TIR (dominio del receptor de Toll/interleuquina 1) implicado en la transducción de la señal (Heumann, Glauser et al. 1998). Se explica así porqué ambos receptores comparten el mismo mecanismo de activación y componentes de la vía de señalización intracelular.

La activación del receptor de TLR4 en respuesta a LPS en macrófagos o del receptor de IL-1 en respuesta a esta citoquina en células Hela, induce una activación de Cot/tpl-2, a los 10 minutos de estimulación, que representa una subida de unas 10 veces respecto a la actividad quinasa endógena basal de la proteína. Esta activación precede a la de ERK1/ERK2 en respuesta a estos estímulos, que alcanza su máximo a los 15 minutos.

Datos previos obtenidos por otros grupos investigadores establecían una relación directa entre la estimulación de la quinasa c-RAF y la activación de macrófagos por LPS (Chakravortty 2001; Moon 2002). Sin embargo, nuestros trabajos demuestran que Cot/tpl-2 es la única MKKK que activa la ruta de ERK1/ERK2 en respuesta a LPS e IL-1.

Además, mientras que el LPS induce en macrófagos una activación de la ruta de ERK1/ERK2 que alcanza su máximo tras 15 minutos de estimulación (Weinstein, Sanghera et al. 1992; Arditi M 1995; Valledor 2000) y que es precedida en el tiempo por el pico de activación de Cot/tpl-2; la activación de ERK1/ERK2 por c-RAF quinasa, en respuesta a la activación de sus receptores específicos, es más rápida alcanzando su valor máximo en torno a los 5-10 minutos.

Estos datos son consistentes con los previamente obtenidos en el ratón knock-out de Cot/tpl-2 en los que la capacidad del LPS para activar la ruta de ERK1/ERK2 en macrófagos peritoneales se encuentra inhibida (Dumitru, Ceci et al. 2000).

En este sistema celular, Cot/tpl-2 es susceptible de ser activado, además de por el LPS mediante receptores TLR4 (Caivano, Rodríguez et al. 2003; Eliopoulos, Wang et al. 2003; Waterfield, Zhang et al. 2003), por ADN-CpG mediante receptores TLR9 (Sugimoto, Ohata et al. 2004; Banerjee 2006). Sin embargo existe cierta controversia en relación al papel de Cot/tpl-2 en la respuesta a ADN-CpG. Así, mientras estudios recientes demuestran que en macrófagos derivados de médula ósea o células B, Cot/tpl-2 es esencial para la activación de ERK1/ERK2 en respuesta a ADN-CpG (Banerjee 2006), en queratinocitos (células que no expresan Cot/tpl-2) o macrófagos peritoneales obtenidos procedentes del ratón knock-out de Cot/tpl-2 la activación de ERK1/ERK2 en respuesta a ADN-CpG no está bloqueda (Sugimoto, Ohata et al. 2004).

Existen además otros estímulos que, en otras líneas celulares, son capaces de activar Cot/tpl-2. En células B, Cot/tpl-2 es la única MKKK capaz de activar la ruta de Erk1/Erk2 en respuesta a estímulos como anti-CD40 o TNF-α (Eliopoulos, 2003).

Todos estos datos indican que la transducción de la señal por parte de los receptores capaces de inducir una activación de Cot/tpl-2 (TLR4, TLR9, CD-40, IL-1R y TNFR) implica por lo general, la participación de moléculas adaptadoras de la familia TRAF involucradas en la activación de NF-kB y las MAPKs siguiendo el esquema:

TLR/IL-1R/CD40/TNFR → MyD88→ IRAK→TRAF2/6→TAK1.

Así, los experimentos realizados con el ratón knock-out para TRAF6 han servido para demostrar que está molécula es esencial para la activación de la ruta de NF-κB y las MAPKs en respuesta a CpG-ADN (TLR9) y LPS (TLR4) (Gohda, 2004).

Además, se ha descrito que una sobreexpresión de TRAF6, capaz de inducir la activación del complejo IKK en células MEF, es suficiente para la activación de Cot/tpl-2 en esta línea celular (Eliopoulos, Wang et al. 2003). Sin embargo, se ha propuesto que una sobreexpresión de TRAF2, que participa en la activación de Cot/tpl-2 en respuesta a TNF-α, no es suficiente para inducir la activación de la quinasa (Eliopoulos, Das et al. 2006).

El grupo de Hostager ha demostrado que, en células B y en respuesta a la activación del receptor de CD-40, el bloqueo de la expresión de TRAF2 no implica una activación deficiente de la ruta de NF-κB ya que TRAF6 puede suplir su función (Hostager 2003).

Por otra parte, nuestros experimentos han demostrado que, en células Hela, TRAF6 es esencial para la activación de la ruta Cot-MKK1-ERK1/ERK2 en respuesta a IL-1 (Rodríguez, Pozo et al. 2006).

TRAF 6 cataliza, junto con una enzima conjugadora de ubiquitina, la adición de una única cadena de poliubiquitina a la quinasa TAK1, lo que induce su activación (Sun, Deng et al. 2004). A su vez TAK1, permite la activación de NF-κB (a través del complejo IKK) y de las distintas cascadas de MAPKs, entre las que se encuentra la vía de las MAP quinasas ERK1/ERK2. (O'Neill 2000; O'Neill 2002).

Independientemente del sistema celular y estímulo utilizado para inducir la activación de Cot/tpl-2, está descrito que, en células no estimuladas, Cot/tpl-2 forma un complejo estable e inactivo con p105 NF-κB y otras proteínas como ABIN-2 (Lang 2004; Sun, Deng et al. 2004; Papoutsopoulou, Symons et al. 2006).

La forma salvaje de la proteína Cot/tpl-2 interacciona con p105 NF-κB. Esta interacción, que fue identificada gracias al sistema de doble hibrido en levaduras (Belich, Salmeron et al. 1999), fue posteriormente corroborada por los resultados de diversos grupos investigadores donde se demostró que en macrófagos en los que la expresión de p105 de NF-κB está bloqueada, no se puede detectar Cot/tpl-2 a menos que se utilicen inhibidores del proteasoma debido a su rápida degradación. En estos experimentos las células presentaban unos niveles normales de ARN mensajero de Cot/tpl-2 (Waterfield, Zhang et al. 2003; Waterfield, Jin et al. 2004).

Por otra parte, estudios de sobreexpresión realizados con p105 NF-κB y la forma salvaje de Cot/tpl-2 indican que Cot/tpl-2 interacciona con p105 NF-κB a través de dos dominios diferentes (Beinke, Robinson et al. 2004). Una de las interacciones se produce a través del dominio carboxilo terminal de la forma salvaje de Cot/tpl-2. Esta interacción explica porqué la interacción entre p105 NF-κB y Cot/tpl-2 protege a la quinasa de degradación.

Una segunda interacción ha sido propuesta como la responsable de impedir la interacción de Cot/tpl-2 con sus sustratos y consecuentemente su fosforilación (Beinke, Robinson et al. 2004; Babu, Jin et al. 2006).

Por otro lado, experimentos de cotransfección demuestran que la proteína ABIN-2 puede interaccionar tanto con Cot/tpl-2 como con p105 NF-κB, pero que de modo preferente forma un complejo ternario con ambas proteínas (Lang 2004). La interacción de ABIN-2 con Cot/tpl-2 ha sido confirmada en macrófagos donde el gen ABIN-2 se encuentra bloqueado. En estos macrófagos, al igual que en macrófagos que no expresan p105 NF-κB, no es posible detectar la proteína Cot/tpl-2 debido a su rápida degradación (Lang 2004; Papoutsopoulou, Symons et al. 2006).

Estos datos indican que tanto la proteína p105 NF-κB como ABIN-2 son requeridas para la formación de un complejo con la forma salvaje de Cot/tpl-2 que protege a la quinasa de la degradación por el proteasoma.

La vida media de la proteína Cot/tpl-2 una vez disociada es extremadamente corta (35 min.). El dominio carboxilo terminal de Cot/tpl-2 salvaje contiene una secuencia de aminoácidos (EMLKRQRSLYIDLGALAGYFNL) denominada degrón, que permite que la proteína sea reco-

nocida por el proteasoma para su posterior degradación. Este degrón confiere inestabilidad a las proteínas que lo contienen y es responsable de la reducción de la vida media de la proteína salvaje de Cot/tpl-2, unas 2,6 veces inferior a la de la forma truncada que carece de esa secuencia. (Gándara y López 2003).

La disociación de Cot/tpl-2 de este complejo es un paso esencial requerido para su activación. El LPS y la IL-1 son capaces de inducir la disociación de Cot/tpl-2 de este complejo (Beinke, Robinson et al. 2004) lo que permite la activación de Cot/tpl-2. Una vez disociada Cot/tpl-2 es degradada rápidamente por el proteasoma.

En nuestros experimentos de estimulación de células Raw con LPS, no detectamos la presencia de p105 en los inmunoprecipitados de la proteína Cot/tpl-2 procedentes de macrófagos no estimulados, probablemente debido a la utilización de un tampón de lisis con una elevada concentración de detergente (1% NP-40) que puede llegar a disociar interacciones entre proteínas (Figura 2 publicación 1).

Así, se detectó una banda única de proteína correspondiente a Cot/tpl-2 tanto en células control como en estimuladas. Además no se observaron diferencias en los niveles de expresión entre ambas bandas, si bien el inmunoprecipitado de macrófagos estimulados con LPS contenía 10 veces más actividad Cot/tpl-2 que el inmunoprecipitado de las células control. Esto indica que si bien el mecanismo de activación de Cot/tpl-2 requiere la disociación de la proteína de p105, es necesaria al menos otra modificación adicional en Cot/tpl-2 para que esta se produzca.

Partiendo de esta hipótesis, nos propusimos estudiar si una fosforilación de Cot/tpl-2 participaba en el proceso de activación de la quinasa.

Nuestros experimentos han demostrado que el tratamiento con la proteína fosfatasa 2A (PP2A) o la proteína fosfatasa 1 (PP1) así como el tratamiento con la tirosina fosfatasa 1B (PP1B) no es capaz de inhibir la activación de Cot/tpl-2 inducida por LPS en los inmunoprecipitados procedentes de extractos de macrófagos (Caivano, Rodríguez et al. 2003). De igual modo, ninguno de los inhibidores específicos de serina/treonina fosfatasas (como el ácido okadáico) o de tirosina fosfatasas (ortovanadato) son capaces de inducir una activación de Cot/tpl-2 en macrófagos intactos.

Sin embargo, la incubación de macrófagos con inhibidores específicos de tirosina quinasas como la herbimicina, impide la activación de Cot/tpl-2 en respuesta a LPS e IL-1 y la subsiguiente activación de ERK1/ERK2. Este mismo compuesto no produce efecto alguno sobre la activación de ERK1/ERK2 en respuesta a PMA.

Estos datos indican que una tirosina quinasa está implicada en el mecanismo de activación de Cot/tpl-2, no sólo en macrófagos en respuesta a LPS, sino también en células Hela y sinoviocitos en respuesta a IL-1.

La incubación de estos tipos celulares con inhibidores como el PP1 y PP2 (específicos de la ruta de las Src quinasas) es capaz de bloquear la activación de ERK1/2 por Cot/tpl-2 en respuesta a IL-1/LPS, mientras que las rutas de p38 y JNK no se ven afectadas. Además el hecho de que el inhibidor PP1 bloquee la activación, pero no la disociación, implica que al menos dos rutas de señalización distintas están involucradas en el mecanismo de activación de Cot/tpl-2.

Existen distintas proteínas quinasas de la familia de Src que pudieran ser activadas en respuesta a LPS o IL-1. Sin embargo tras realizar experimentos utilizando la técnica de los ARNsi para los distintas tirosinas quinasas pertenecientes a la familia de Src, no se detectó ningún ARNsi que fuera capaz, por si solo, de impedir la fosforilación de GST-ERK2 inducida por IL-1. Por tanto, existe la posibilidad de que se requiera la actuación de más de una quinasa de la familia de Src para activar Cot/tpl-2.

Además, la utilización de inhibidores de otras familias de tirosina quinasas como los tirfóstidos AG126, AG490 (tirfostina B42), AG957 o AG1296, no ejercen efecto alguno sobre la activación de ERK1/2 mediada por Cot/tpl-2 en respuesta a IL-1 en células Hela o en macrófagos inducida por LPS.

Según estos datos podemos afirmar que, al menos en respuesta a la estimulación por IL1 y LPS, Cot/tpl-2 se activa mediante un mecanismo que involucra la actividad de una(s) quinasa(s) de la familia de Src (Rodríguez, Pozo et al. 2006). De igual modo que una(s) quinasa(s) de la familia de Src se requiere para la activación de Cot/tpl-2 en respuesta a LPS e IL-1, la tirosina quinasa Syk se requiere para la activación de Cot/tpl-2 en respuesta a TNF-α (Eliopoulos, Das et al. 2006). En

este sistema el tratamiento de macrófagos, células B, Jurkat o Hela con un inhibidor específico de la tirosina quinasa Syk (piceatanol) bloquea la activación de ERK1/ERK2 en respuesta a TNF-α mientras que la utilización del inhibidor específicos de Src quinasas (PP2) no ejerce efecto alguno sobre la activación de ERK1/ERK2.

Así estos estudios refuerzan la teoría de que, independientemente del sistema celular utilizado, las distintas señales extracelulares utilizan distintas tirosinas quinasas para la activación de una misma ruta de señalización. Aunque no podemos excluir la posibilidad de que la activación de Cot/tpl-2 implique la activación secuencial de al menos 2 tirosinas quinasas mediante un mecanismo que induzca una activación de Syk y posteriormente de una quinasa de la familia de Src o viceversa en respuesta a la estimulación por IL-1 y/o LPS.

Existen varios trabajos que afirman que la fosforilación del residuo de treonina 290 de Cot/tpl-2 es requerida para la activación de la quinasa. Si bien es cierto que existe cierta controversia acerca de si esta fosforilación se produce o no en respuesta a LPS (Luciano, Hsu et al. 2004; Cho et al 2005; Cho et al 2005; Stafford et al 2006).

Estudios más recientes han confirmado que Cot/tpl-2 requiere, al menos en respuesta a IL-1, además de la fosforilación del residuo de serina 290, una autofosforilación del residuo de serina 62 para ser completamente activa (Stafford, Morrice et al. 2006). Sin embargo, se desconoce cuál es la quinasa encargada de inducir la fosforilación de la serina 290, aunque se ha establecido que su actividad es independiente de la activación del complejo IKK (Cho, Melnick et al. 2005; Cho y Tsichlis 2005; Stafford, Morrice et al. 2006).

Además, el grupo de Weiss ha demostrado que la co-transfección en células HEK de Cot/tpl-2 y proteína quinasa B (PKB/AKT) induce la fosforilación del residuo de serina 400 en el extremo carboxilo terminal de Cot/tpl-2 salvaje (Kane, Mollenauer et al. 2002). Esta fosforilación induce la capacidad de Cot/tpl-2 de activar la ruta de NF-κB, sin embargo no la capacidad de Cot/tpl-2 para activar ERK1/ERK2.

Por otro lado, y aunque la activación de PKB/Akt se realiza mediante un mecanismo que involucra a la PI3K, nuestros datos indican que la PI3K no está implicada en la activación de

Cot/tpl-2, medida como MKKK1, en respuesta a LPS o IL-1 De hecho la incubación de macrófagos Raw con LY294002, un potente inhibidor de la PI3K, no es capaz de bloquear la activación de Cot/tpl-2 en respuesta a estos estímulos. Así pues, la fosforilación del residuo de serina 400 no tiene efecto sobre la capacidad de Cot/tpl-2 para activar ERK1/ERK2 en respuesta a LPS/IL-1.

Así pues, se postula que la activación de Cot/tpl-2 requiere, además de la disociación del complejo p105 NF-κB, una fosforilación del residuo de serina 290 por una proteína quinasa de naturaleza desconocida (aunque según nuestros datos podría ser una quinasa activada por un miembro de la familia de Src quinasa) y una autofosforilación del residuo de serina 62. Bien es cierto que se desconoce cuales son los requerimientos exactos para la activación de la proteína una vez disociada del complejo p150 NF-κB/Cot, incluso puede que estos varíen de un tipo celular a otro.

La disociación de Cot/tpl-2/p105 NF-κB, que es esencial para la activación de Cot/tpl-2, da lugar posteriormente a su degradación. Se ha descrito que los niveles de ARNm de Cot/tpl-2 quinasa se encuentran incrementados en respuesta a IL-1 de tal modo que los mismos estímulos que inducen la degradación de Cot/tpl-2 estimulan su síntesis asegurando así el recambio proteico (Chan, Chedid et al. 1993).

Por otro lado, hemos establecido que la 15-d-PGJ_2, uno de los ligandos naturales de los receptores activados por proliferadores peroxisomales tipo-γ (PPAR-γ), es capaz de bloquear la activación de Cot/tpl-2 en respuesta a LPS (Caivano, Rodríguez et al. 2003).

Los PPARs son una familia de receptores nucleares que han sido relacionados con el control de la inflamación y, más concretamente, con la regulación de producción de citoquinas proinflamatorias.

De hecho algunos ligandos de PPAR-γ inhiben la producción de TNF-α, IL-1α, IL-6 y óxido nítrico sintasa en monocitos, macrófagos activados o células endoteliales (Ricote 1998; Alleva 2002; Straus 2000).

No obstante, los efectos de estos ligandos no siempre implican la activación de sus receptores

asociados como ocurre en este caso. Nuestros experimentos demostraron que la 15-d-PGJ_2 era capaz de bloquear la activación de Cot/tpl-2 en respuesta a LPS tanto *in vivo* como *in vitro* lo que indicaría que la capacidad de la prostaglandina para inhibir a Cot/tpl-2 no requiere la participación del receptor PPAR–γ y por tanto que sus efectos como antiinflamatorio no implican la activación de su receptor específico. De hecho la rosiglitazona un ligando sintético de los PPARs, no es capaz de bloquear la activación de Cot/tpl-2.

Además de esta molécula, estudios recientes han descubierto que ciertos carbonitrilos de síntesis química son también capaces *in vitro* de inhibir la activación de Cot/tpl-2 (Hu 2006; Gavrin 2005).

Todos estos datos indican que Cot/tpl-2 endógena participa en la señalización celular en respuesta a diversas señales extracelulares y como consecuencia de ello controla o participa en la respuesta celular a dichas señales que en muchos casos puede ser mimetizada por sobreexpresión tanto de la forma salvaje como de la forma truncada/modificada.

Tanto Cot como tpl-2 fueron identificados en una forma oncogénica y, durante años, se pensó que el proto-oncogen Cot/tpl-2 estaba implicado en las rutas de transducción de señales intracelulares de proliferación. De hecho tpl-2, la forma murina de Cot, fue identificada en una versión modificada/truncada debido a su capacidad para inducir linfomas (Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993).

Además, ciertos estudios habían establecido una relación directa entre la sobreexpresión de Cot/tpl-2 y el incremento en la trascripción de citoquinas proinflamatorias como IL-2 y TNF-α en células T (Ballester, Tobena et al. 1997; Ballester, Velasco et al. 1998; Tsatsanis, Patriotis et al. 1998; Tsatsanis, Patriotis et al. 1998; Eliopoulos, Wang et al. 2003).

Al sobreexpresar tanto la forma salvaje de Cot/tpl-2 como su forma truncada/modificada, se produce una activación de distintas rutas de MAPKs como ERK1/ERK2, JNK (Salmeron, Ahmad et al. 1996), p38γ y ERK5 (Chiariello, Marinissen et al. 2000). Esta sobreactivación produce a su vez la activación de diversos factores transcripcionales como AP-1 (Ballester, Velasco et al. 1998; Hagemann, Troppmair et al. 1999; Chiariello, Marinissen et al. 2000). De hecho, se ha propuesto

que la capacidad de Cot/tpl-2 de producir transformación celular se debe a su capacidad para activar a AP-1 (Chiariello, Marinissen et al. 2000).

Diversos grupos investigadores han demostrado la capacidad de Cot/tpl-2 sobreexpresada para inducir la activación de NF-κB mediante un mecanismo mediado por la quinasa NIK. Esta quinasa incrementa la actividad del complejo IKK, lo que induce a la degradación de IκBα (Tsatsanis, Patriotis et al. 1998; Belich, Salmeron et al. 1999; Lin, Cunningham et al. 1999; Sebald, Mattioli et al. 2004). Esta hipótesis es corroborada con observaciones más recientes que demuestran que, en organismos adultos afectados por la leucemia de células T inducida por el virus HTLV-I, la actividad incrementada de Cot/tpl-2 está relacionada con una desregulación de la actividad de NF-κB (Babu, Waterfield et al. 2006).

También se ha descrito que Cot/tpl-2 vía ERK1/ERK2, incrementa de la actividad de los factores de transcripción CREB (Eliopoulos, Dumitru et al. 2002) y E2F (Velasco-Sampayo y Alemany 2001).

Sin embargo, el hecho de que el ratón knock-out de Cot/tpl-2 sea resistente a la patología inducida por LPS/D-Galactosamina debida a la baja producción de TNF-α indica que, en términos fisiológicos, Cot/tpl-2 endógena podría estar más relacionada con la inmunidad innata que con las señales proliferativas (Dumitru, Ceci et al. 2000).

En esta línea, nosotros hemos demostrado que Cot/tpl-2 está implicada en la regulación de la expresión de IL-8 y MIP-1β en respuesta a IL-1, dos citoquinas que juegan un papel de importancia en el desarrollo de procesos inflamatorios. Así, IL-8 es una quimocina proinflamatoria que actúa como quimoatrayente de neutrófilos, mientras que la quimocina MIP-1β es un potente activador de macrófagos.

Está también establecido que, en macrófagos en respuesta a LPS, Cot/tpl-2 quinasa regula la expresión de COX-2 mediante un mecanismo que, a través de ERK1/ERK2, induce la activación de las quinasas p90Rsk y Msk1. Estas quinasas son las encargadas de fosforilar el factor de transcripción CREB implicado en la transcripción de COX-2 (Eliopoulos, Dumitru et al. 2002).

Además una sobreexpresión de Cot/tpl-2 en células Jurkat, incrementa la actividad transcripcional del promotor de COX-2 al activar los elementos de respuesta a NFAT distales (-105/-97) y proximales (-76/-61) (de Gregorio, Iniguez et al. 2001).

Nuestros estudios demuestran como la forma truncada de Cot/tpl-2 (constitutivamente activa) induce un cambio en el proteoma de las células Hela, en las que se produce un incremento en los niveles de expresión de COX-2. Actualmente se desconoce el mecanismo por el cual Cot/tpl-2 induce un incremento en los niveles de COX-2 en células Jurkat. Sin embargo, si que ha establecido la implicación de las vías de NF-κB y ERK1/ERK2 en el incremento de los niveles de COX-2 en células Hela (Chun y Surh 2004; Wu 2005; Telliez, Furman et al. 2006) y Cot/tpl-2 truncada es capaz de activar ambas vías.

Recientemente, se ha establecido una relación directa entre los niveles de COX-2 y la tumorogénesis. Así se ha relacionado la expresión de COX-2 con una mayor motilidad celular, capacidad invasiva y con la producción de metaloproteasas en células de cáncer de mama (Larkins, 2006; Singh, Berry et al. 2005; Hiraga, Myoui et al. 2006, Sinicrope).

Esto refuerza la idea de que fenómenos como la inflamación y la invasión y migración celular comparten bases moleculares.

En este sentido nuestros experimentos han demostrado que la IL-1, una potente citoquina relacionada con procesos inflamatorios, es capaz de inducir mediante un mecanismo que implica la activación de Cot/tpl-2, un aumento de la expresión del ARNm de distintas metaloproteasas (MMPs).

Las MMPs son unas endopeptidasas dependientes de zinc que juegan un papel esencial en la migración e invasión celular, su desregulación está asociada a fenómenos de malignidad celular.

De hecho se ha establecido una correlación entre la expresión de MMP1, MMP9, MMP10 y MMP11 y el aumento de la maglinidad y la capacidad invasiva en distintas líneas celulares (Pulyaeva, Bueno et al. 1997; John y Tuszynski 2001; Fridman, Toth et al. 2003; Mercapide, López De Cicco et al. 2003; Thorns, Walter et al. 2003; Kousidou, Roussidis et al. 2004; Sato,

Takino et al. 2005; Sossey-Alaoui, Ranalli et al. 2005; Turpeenniemi-Hujanen 2005; Yuan 2005; Tolboom 2002).

Nosotros hemos establecido que, en células Hela y en respuesta a IL-1, la inhibición de la expresión de la proteína Cot/tpl-2 endógena, mediante una mezcla de ARNsi específicos, bloquea la inducción de la expresión del ARNm de estas proteasas. Sin embargo, se desconocen cuales son los mecanismos moleculares exactos por los que Cot/tpl-2 induce la activación de la transcripción de estas cuatro metaloproteasas.

Se ha descrito, que la activación de la vía de señalización de ERK1/ERK2 juega un papel relevante en la activación de los promotores de los genes de MMP1, MMP9, MMP10 y MMP11 puesto que ERK1/ERK2 es capaz de inducir la activación de factores de transcripción como AP-1 y Ets (Brauchle, Gluck et al. 2000; Lakka 2002; Tanimura, Asato et al. 2003; Ulku 2003; Moon, Cha et al. 2004; Ramos, Steinbrenner et al. 2004; Fromigue 2003). Este hecho explicaría, al menos en parte, como la inhibición de Cot/tpl-2 bloquea la expresión de los ARNm estas metaloproteasas.

Cabe destacar que la expresión de estas 4 metaloproteasas implica la activación de mecanismos de transducción de señales distintos. Así mientras la activación de ERK1/ERK2 junto con la activación de JNK y NF-κB podría explicar un aumento de la transcripción de los ARNm de MMP1 y MMP10, no explicaría la activación de MMP9 y MMP11 ya que un aumento de la expresión de Cot/tpl-2 truncada no induce un aumento en la expresión de estas metaloproteasas.

Además nuestros experimentos demuestran que, al menos en células HeLa en las condiciones experimentales utilizadas, Cot/tpl-2 endógena posee cierta actividad intrínseca y que esta actividad está implicada en migración celular. De hecho, un incremento anormal de la actividad quinasa de Cot/tpl-2 aumenta la migración celular.

La migración celular es un proceso secuencial y complejo donde los componentes se mantienen en un equilibrio dinámico. Para que una célula migre, se requiere la reorganización de los componentes del citoesqueleto y la formación de nuevas uniones adherentes que aportaran la fuerza tractora necesaria para producir la translocación celular.

Diversos estudios han establecido una relación directa entre la activación de las rutas de ERK1/ERK2 y JNK, bien por activación fisiológica o por desregulación de la actividad, con fenómenos de migración celular (Reddy, Nabha et al. 2003; Huang, Jacobson et al. 2004).

Estudios anteriores a nuestro trabajo postulan que las MKKK que regulan fenómenos de migración e invasión celular son respectivamente, RAF (Ehrenreiter, Piazzolla et al. 2005; Rajalingam, Wunder et al. 2005) y MEKK1 (Yujiri, Ware et al. 2000; Xia y Karin 2004; Deng, Chen et al. 2006). Nuestros estudios demuestran sin embargo que una sobreactivación de la MKKK Cot/tpl-2, que induce una activación de ERK1/ERK2 y JNK, también produce un aumento en la capacidad invasiva y migratoria celular.

También demostramos que la inhibición de la actividad endógena de Cot/tpl-2 produce una disminución de los niveles de invasión y migración celular. Esta disminución en la migración/invasión correlaciona con una reducción en los niveles basales de fosfo ERK1/ERK2. De hecho el tratamiento de células control o células que sobre-expresan Cot/tpl-2 truncada con un inhibidor de la activación de ERK1/ERK2 bloquea totalmente su capacidad de migración (datos no mostrados).

El trabajo realizado durante el desarrollo de la Tesis Doctoral ha permitido establecer, la implicación de COX-2 en la regulación de la migración e invasión celular. Así, la utilización de un inhibidor específico de COX-2 (NS-398) es capaz de bloquear la migración de células que expresan la proteína Cot truncada. Además el producto de COX-2, PGE_2 incrementa la migración de células control equiparándola con los niveles de migración correspondientes a unas células que tienen los niveles de COX-2 elevados como consecuencia de la sobreexpresión de Cot/tpl-2 truncada.

Todos estos datos indican que el incremento en los niveles de COX-2 juega un papel esencial en la mayor capacidad de migración que presentan las células que tienen una actividad Cot/tpl-2 patológicamente incrementada.

Sin embargo la adición de PGE_2, a células transfectadas con los ARNsi de Cot/tpl-2 no incrementa la capacidad invasiva de estas células. Así, podemos inferir que la proteína Cot/tpl-2 endógena debe controlar eventos distintos a los regulados por esta prostaglandina. Aún más, en células Hela,

la estimulación con PGE_2 no produce un aumento en los niveles de fosforilación de ERK1/ERK2 y, como hemos mencionado anteriormente, el estado de fosforilación de ERK1/ERK2 tiene un papel esencial en la migración celular. Además, la adición de PGE_2 a células transfectadas con el ARNsi de Cot/tpl-2 no es capaz de revertir el incremento en los niveles de Rho-GTP observado en estas células.

Pese a que Cot/tpl-2 endógena parece no estar implicada en fenómenos de adhesión celular, hemos demostrado que una sobreexpresión de la forma truncada de Cot/tpl-2 induce una disminución en los niveles de adhesión de células Hela independientemente del substrato utilizado para el ensayo. Así, las células que sobreexpresan Cot/tpl-2 presentan unos mayores niveles de PAI-1 (inhibidor del activador del plasminógeno-1). De hecho, estudios recientes demuestran que un aumento de la activación de las rutas de ERK1/ERK2 y JNK está relacionado con el aumento de la transcripción del gen de PAI-1 (Reddy, Nabha et al. 2003; Providence y Higgins 2004; Buchwalter, Gross et al. 2005). Considerando que la proteína PAI-1 disminuye la capacidad de las células a adherirse a distintos tipos de matrices extracelulares (Czekay, Aertgeerts et al. 2003) cabe la posibilidad de que la menor capacidad adhesiva presente en células que sobreexpresan Cot/tpl-2 truncada sea debida a la regulación que Cot/tpl-2 truncada ejerce sobre la expresión de la proteína PAI-1.

Las células que sobreexpresan Cot/tpl-2 truncada presentan, comparadas con células en las que la expresión de Cot/tpl-2 endógena se encuentra inhibida y células control, características típicas de células con alta capacidad invasiva como una disminución en el número de fibras de estrés, un mayor número lamelipodios y una polarización de los microtúbulos y filamentos intermedios.

Cabe señalar que mientras que las células control y las transfectadas con los ARNsis de Cot/tpl-2 presentan diferencias significativas en los niveles de migración e invasión celular, no se observan cambios significativos en la organización de los microtúbulos o de los filamentos intermedios.

Está establecido que el control de la formación de las fibras de estrés está regulado por Rho (Small 2002)mientras que otros miembros de la familia de Rho como Rac o Cdc42 están implica-dos en procesos como la formación del lamelipodio y la formación del filopodio respectivamente.

Además diversos estudios demuestran la importancia de los efectores de estas Rho GTPasas en los procesos implicados en la migración celular. Así se ha establecido, que la activación de Rho induce la formación de fibras de estrés y contactos focales mediante un mecanismo que implica a la quinasa ROCK (Riento, Guasch et al. 2003; Riento y Ridley 2003).

De acuerdo con estos datos, nuestros estudios confirman que la activación de ROCK es esencial para la inducción de la migración celular independientemente de los niveles de expresión de Cot/tpl-2. Así la utilización de un inhibidor específico de esta quinasa (Y-27632) es capaz de bloquear la migración tanto en células control como en células que sobre-expresan Cot/tpl-2.

Se ha descrito que una cierta activación basal de Rho es necesaria para la producción de la fuerza adhesiva requerida para inducir la motilidad celular y como un exceso de activación de Rho inhibe la migración celular. En esta línea, diversos estudios corroboran que un aumento de la actividad Rho A induce un aumento en la formación de fibras de estrés, una disminución en el número de protusiones y por ende una disminución de la motilidad celular debido a la formación de fuertes contactos focales maduros (Arthur 2001; Ridley 2001 b; Ridley 2001a).

En consonancia con estas teorías nuestros estudios demuestran que en el caso de las células transfectadas con ARNsi de Cot/tpl-2 los niveles de Rho A/B/C activa experimentan un aumento significativo con respecto a los presentes en células transfectadas con la forma truncada de Cot/tpl-2 o células control. Además la utilización de un anticuerpo específico contra paxilina demuestra que las células transfectadas con ARNsi de Cot/tpl-2 presentan una mayor tinción para esta proteína lo que indica que estas células presentan un mayor número de adhesiones focales maduras (Hagel, George et al. 2002; Zaidel-Bar, Ballestrem et al. 2003) y por tanto menor motilidad. Este dato es corroborado por el hecho de que las células transfectadas con ARNsi apenas presenten complejos de ARP2/3 lo que significa un menor número de lamelipodios (Machesky, Reeves et al. 1997; Pollard y Borisy 2003).

Esto datos explicarían, al menos en parte, por que las células en las que Cot/tpl-2 se encuentra inhibida apenas presentan capacidad invasiva o migratoria comparadas con células control.

Todos estos datos corroboran la importancia de Cot/tpl-2 en los procesos de migración e invasión celular aportando datos novedosos sobre la implicación de Cot/tpl-2 en procesos de malignidad y agresividad de tumores.

Durante los últimos 15 años el número de evidencias que relacionan mutaciones del gen de Cot con la inducción de malignidad se ha ido incrementando considerablemente (Miyoshi, Higashi et al. 1991; Chan, Chedid et al. 1993; Makris, Patriotis et al. 1993; Patriotis, Makris et al. 1993; Erny, Peli et al. 1996; Ceci, Patriotis et al. 1997; Sourvinos, Tsatsanis et al. 1999; Chiariello, Marinissen et al. 2000; Eliopoulos, Davies et al. 2002; Gándara 2003; Clark, Reynolds et al. 2004; Christoforidou, Papadaki et al. 2004; Babu, Waterfield et al. 2006).

Durante el desarrollo de esta Tesis Doctoral hemos demostrado que la capacidad de Cot/tpl-2 para inducir un incremento de la migración celular se debe a su implicación en los mecanismos de regulación de diferentes pasos implicados en este proceso.

Por otro lado, se ha puesto de manifiesto que existe una relación entre inflamación e invasión demostrando que ambos procesos comparten bases moleculares (Chakraborti, Mandal et al. 2003; Karin 2005; Aggarwal 2006).

Nosotros hemos demostrado que la MKKK Cot/tpl-2, que posee un papel establecido en inmunidad innata (Dumitru, Ceci et al. 2000; Eliopoulos, Dumitru et al. 2002; Caivano, Rodríguez et al. 2003; Eliopoulos, Wang et al. 2003; Waterfield, Zhang et al. 2003; Sugimoto, Ohata et al. 2004; Das, Cho et al. 2005; Rodríguez, Pozo et al. 2006), también regula la invasión celular. Así, Cot/tpl-2, que siempre ha sido propuesta como una molécula diana interesante en el desarrollo de medicamentos antiinflamatorios, podría ser además utilizada para desarrollar drogas que bloqueen la invasión celular.

CONCLUSIONES

Las conclusiones más relevantes extraídas de este trabajo son:

1. Cot/tpl-2 es la única MAP quinasa quinasa quinasa que activa la ruta de ERK1/ERK2 en respuesta a LPS y IL-1.

2. El mecanismo de activación de Cot/tpl-2 es dependiente de la activación de la molécula adaptadora TRAF6→IKKβ y requiere además la actuación de una(s) tirosina quinasa (s) de la familia de Src.

3. La 15-d-PGJ_2 es capaz de bloquear *in vitro* la actividad quinasa de Cot/tpl-2.

4. Cot/tpl-2 media un aumento de los ARNm de diversas moléculas como el MIP-1β, la IL-8.

5. Cot/tpl-2 está implicada en la regulación de procesos celulares como la invasión y migración celular debido a su capacidad para regular la activación de Rho y la expresión de COX-2. Además, Cot/tpl-2 media un aumento del ARNm de las metalopreteasas MMP1, MMP9, MMP10 y MMP11.

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